Immunotherapeutic strategies in patients with solid malignancies

Voskens, Caroline Joanna

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Crossing the line between scientific possibility and clinical reality

Caroline J. Voskens

Increasing clinical data suggests that eradication of human cancer may best be accomplished by concurrent activation of the various components of the immune system. This may be achieved by combining cancer treatment modalities which are able to enhance antitumor immune responses within the innate or adaptive immune system, by combing cancer treatment modalities, which simultaneously activate both innate and adaptive immunity or by combining treatment modalities, which stimulate tumor-specific immunity and target co-stimulatory pathways. At first, most treatment strategies prioritized the stimulation of CTL responses based on their ability to directly and specifically kill tumor cells. Currently, most immunotherapeutic strategies in patients with solid tumors are geared towards direct elimination of tumor cells or direct activation of the adaptive immune system, whereas relatively few are intended to stimulate co-signaling pathways or the innate immune system. For that reason, this thesis focuses on CD137-mediated co-stimulation (part A) and NK cell-based immunotherapy (part B) and their therapeutic potential in the treatment of solid malignancies. In addition, results of a clinical pilot study testing a MAGE and HPV peptide-based vaccine, which was shown to stimulate both CTL and T helper cell responses in murine models are presented and discussed (part C). Overall, therapeutic strategies discussed in this thesis provide a foundation on which to add additional immunotherapies to facilitate improved treatment of select solid tumors.

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"God, give me grace to accept with serenity
the things that cannot be changed,
Courage to change the things
which should be changed,
and the Wisdom to distinguish
the one from the other."

Serenity Prayer by Reinhold Niebuhr

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Stellingen behorend bij het proefschrift

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1. Immobilized Fc enhances the expression of CD137 on IL-2 stimulated human NK cells and expression levels are dependent on patterns of Fc glycosylation (this thesis)

2. CD137 expressing human NK cells play a role in immunomodulation rather than exhibiting direct cytotoxic functions (this thesis)

3. CD137-CD137L interactions promote human B cell proliferation and survival (this thesis)

4. *Ex vivo* NK cell expansion in the presence of exogenous IL-2 and CD137L/IL-15 expressing K562 cells turns the NK receptor balance towards activation resulting in natural cytotoxicity and ADCC against autologous gastric tumor cells (this thesis)

5. Polymorphisms at amino acid position 158 of the Fc receptor are associated with antigen-dependent and antigen-independent NK cell mediated cytotoxicity (this thesis)

6. The disparity of CD137 expression and function between human and murine NK and B cells challenges the use of murine-based disease models for the evaluation of CD137 mediated immune regulation (this thesis)

7. Applying immune-related response criteria on previously conducted clinical studies will likely re-identify various immunotherapeutic strategies with proven favorable toxicity profiles and potential benefit for patients with solid tumors

8. „It is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change"
Attributed to Charles R. Darwin

9. "Pride: Having it makes giving anything less than your best an impossibility. Conversely, knowing you have given your best makes the failure bearable"
Marshall Strome

10. Vasthouden betekent loslaten

11. "The future belongs to those who can see opportunities before they get obvious"
Oscar Wilde

For Siddhartha

Paranimfen: Drs. K. Neuteboom
Mr. E. Schulting

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Introduction and outline of the thesis

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SYNTHETIC PEPTIDE-BASED CANCER VACCINES: LESSONS LEARNED AND HURDLES TO OVERCOME

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Cancer immunotherapy is a form of cancer treatment, which holds great promise. Its main premise is to stimulate natural occurring immune responses or to facilitate *de novo* immune responses directed against tumor cells. Despite their recognized therapeutic potential in murine models, most immunotherapeutic strategies have demonstrated little clinical efficacy when tested in phase I/II clinical trials (1). Recent reviews have reported that objective tumor regression in cancer patients is rare when measured by strict Response Evaluation Criteria In Solid Tumors (RECIST) criteria (2). In many of the clinical studies, the tumors failed to regress despite robust antigen-specific immune responses as measured by *ex vivo* laboratory techniques. These findings have led to two main questions. First, why is there little correlation between antigen-specific immune response and outcome among clinical studies? Second, how can we improve immunotherapeutic strategies to optimize therapeutic efficacy?

Main characters of the immune system

The first line of defense against tumor cells is mediated by the innate immune system. This response is quick, non-specific, short-lived and mediated by neutrophils (including granulocytes and eosinophils), macrophages, natural killer (NK) cells, γδ T cells, monocytes and dendritic cells. During its mode of action, the innate immune system also activates the adaptive immune system. This second line of defense is slow, specific, long-lasting and generally mediated by T and B cells.

Innate immune system

NK cells

NK cells are thought to be the most important cells of the innate immune system that play a role in antitumor immunity. They were identified more than 30 years ago as a population of lymphokine activated killer cells that showed the ability to kill tumor cells *in vitro* in the absence of prior immune sensitization of the host (3-6). Their biologic activity is controlled by a complex repertoire of surface receptors which, upon engagement by ligands on a target cell, signal either an inhibitory or activating (cytotoxic) response (7). The major inhibitory and activating receptors are products of germ line genes encoding killer cell immunoglobin-like receptors (KIRs) and in an autologous environment, inhibition of NK cell cytotoxic activity is dominant and governed by epitopes on self HLA class I alleles. This functional competency is also known as licensing (8). In general, cytotoxic activity is triggered when the target cell lacks expression of some or all HLA class I molecules; the basis for the "missing self" hypothesis (9). Importantly, NK cells express Fc-receptors, which enable them to mediate tumor cell killing through a

mechanism known as antibody-dependent cellular cytotoxicity (ADCC) (10,11). In addition, NK cells may amplify the adaptive immune response through the production of cytokines (e.g. Interferon-γ and Interleukin-2) which directly enhances the antigen presentation function of dendritic cells (12).

Dendritic cells

Dendritic cells (DC) possess an unique antigen presenting capacity since they are able to take up and process tumor-derived antigens (13). After processing, the tumor-derived antigens are presented in the context of HLA class I and II molecules expressed on the DC to T cells (14). In addition, DC provide co-stimulatory signals necessary to induce tumor-specific T cell expansion (15). Importantly, only fully mature DC are able to boost tumor-specific immunity, since lack of DC maturation is associated with T cell tolerance (16).

Adaptive immune system

T cells

T cells are generally separated into $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ T helper cells. CTL recognize tumor-specific peptides presented in the context of HLA class I molecules expressed on most nucleated cells in the body, whereas T helper cells mainly recognize tumor-specific peptides presented in the context of HLA class II molecules expressed on DC. CTL are able to directly mediate antitumor directed immune responses. In contrast, the direct interaction of T helper cells with dendritic cells results in the activation and maturation of these cells and facilitates the effective presentation of tumor-specific antigens to CTL (17-19). Furthermore, tumor-specific T helper cells are required for the generation of tumor-specific memory T cell responses (20,21). Various subsets of $CD4^+$ T helper cells have been identified including (i) T helper 1 (Th-1) cells, (ii) T helper 2 (Th-2) cells and (iii) IL-17 producing T helper (Th-17) cells. The secretion of Th-1 specific cytokines (e.g. IFN-γ, IL-2) by Th-1 cells further supports induction of T cell mediated antitumor immunity. For example, the release of IFN-γ by activated Th-1 cells activates antigen-presenting cells to up-regulate HLA class I and co-stimulatory molecules which augment overall antigen presentation to CTL (22). In addition, the production of IL-2 directly stimulates CTL growth and proliferation (23) and it is recently reported that Th-1 cells rescue CTL from activation-induced-cell-death (AICD) upon antigen encounter (24). Importantly, Th-1 cells not only provide help to CTL, they also demonstrate direct cytotoxic activity against tumor cells in several studies (25-29). There is evidence that some tumors (e.g. melanoma, lung cancer, breast cancer and osteosarcomas) express HLA class II which facilitates recognition and direct killing by Th-1 cells (30). Th-2 specific

cytokines (e.g. IL-4, IL-5, IL10 and IL-13) secreted by Th-2 cells establish delayed-type-hypersensitivity reactions in the tumor microenvironment, thereby recruiting tumoricidal macrophages and degranulating eosinophils to the tumor area (31), whereas Th17 cells are thought to play an important role in inflammation (32). Finally, regulatory T cells (Treg) are described. Treg are known to suppress $CD8^+$ and $CD4^+$ T cells and are critical for the maintenance of self tolerance (33).

B cells

Upon T cell mediated activation, B cells start producing antigen-specific antibodies which are able to directly recognize and destroy tumor cells (34,34,35). Furthermore, like DC, B cells are able to capture, process and present tumor-specific antigens in the context of HLA class II molecules (36), which further enhances antitumor immunity. Moreover, B cells regulate immunosuppression through the secretion of Interleukin-(IL)10 (37), the expression of death-inducing ligands (e.g. Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)) (38,39), the expression of costimulatory molecules with inhibitory properties (e.g. PD-Ll and PD-L2) (40,41) and the secretion of regulatory antibodies (42).

Co-stimulation

Long-lasting antitumor immune responses require T cell activation. In turn, effective T cell activation depends on two subsequent signals. First, T cell receptor (TCR) ligation with antigen presented by HLA molecules expressed on DC followed by so called "co-stimulation" mediated through defined co-stimulatory receptor-ligand interactions (43). Co-stimulation results in T cell proliferation, differentiation and survival, while lack of co-stimulation induces T cell anergy, T cell deletion and immune tolerance (44). The most studied co-stimulatory molecules belong to the B7 family (45) or the TNF family (46) and depending on receptor-ligand interaction, may signal either activation, inhibition or both (tables 1 and 2). The ability of co-stimulating molecules to modulate T and B cell function and thus antitumor immune responses, has stimulated significant interest in manipulating these pathways with therapeutic intent. As a result, several humanized antibodies targeting various co-stimulatory pathways are currently under clinical evaluation.

B7 family

CD28

The CD28 receptor is expressed on T cells and recognizes the ligands CD80 and CD86 (also called B7-1 and B7-2) expressed on DC. Ligation promotes T cell proliferation and T cell survival (47) as well as the production of cytokines required for B cell activation (48). Anti-CD28 targeting initially held great promise, although this enthusiasm was dramatically tempered after administration of an anti-CD28 super agonistic antibody to healthy volunteers in 2006. Its infusion resulted in a massive release of pro-inflammatory cytokines inducing multi-organ failure (49) and to date, no further clinical testing has been approved.

CTLA-4 (CD152)

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is expressed on activated T cells and, like CD28, interacts with CD80 and CD86 expressed on DC. However, binding triggers an inhibitory signal resulting in T cell inhibition. Based on binding affinity, CTLA-4 competes favourably with CD80 and CD86 and therefore regulates ongoing antitumor immune responses. Recently, a fully humanized anti-CTLA-4 antibody (ipilimumab, Yervoy®) has been approved for the treatment of unresectable or metastatic melanoma.

Table 1 Receptors and ligands belonging to the B7 family

receptor	expression	ligand	expression	signal	ref
CD28	T cells	B7-1 (CD80)	DC	activation	39
		B7-2 (CD86)	DC	activation	
CTLA-4 (CD152)	T cells	B7-1 (CD80)	DC	inhibition	157
		B7-2 (CD86)	DC	inhibition	
PD-1 (CD279)	T cells, B cells; NKT cells; DC; monocytes;	B7-H1 (PD-L1)	T cells; tumor cells	inhibition	51
		B7-DC (PD-L2)	DC; macrophages	activation; inhibition	
ICOS	T cells	B7-H2 (L-ICOS)	DC; tumor cells; endothelial cells	activation	158 159
unknown	n/a	B7-H3	tumor cells	activation; inhibition	160
unknown	n/a	B7-H4	tumor cells	inhibition	161 162

Ref indicates reference; DC, dendritic cell; CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed death 1; NKT, natural killer-like T; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2; ICOS, inducible T cell co-stimulator; L-ICOS, inducible T cell co-stimulator ligand

Ipilimumab enhances pre-existing immune responses, including antitumor immune responses which results in prolonged median survival and the induction of objective clinical responses in 10-15% of treated patients (50). However, its mode of action is also likely to break tolerance (51) and immune-mediated adverse reactions (irEAs) are observed in up to 60% of treated patients (50).

(PD-1) CD279

The ligands for programmed death 1 (PD-1) are the B7 family members PD-L1 (B7-H1) and PD-L2 (B7-DC) (45). While PD-L1 is expressed on various cell types (52-55), including immune cells and tumor cells, PD-L2 is mainly found on activated macrophages and DC (56). PD-1 is expressed on activated T cells, B cells, DC, activated natural killer-like T (NKT) cells and monocytes (40,57). PD-1-PD-L1/PD-L2 interactions induce T and B cell inhibition (58). In addition, PD-1-PD-L1/PD-L2 interactions may also directly mediate an inhibitory signal into PD-L1/PD-L2 expressing DC or tumor cells (reverse signaling), which may render the latter resistant to T cell-mediated cytotoxicity (59). First in-human clinical studies demonstrated evidence of antitumor activity and limited toxicity of a fully human IgG4 anti-PD-1 (60,61) and a fully human IgG4 anti-PD-L1 blocking antibody (62) in select refractory or relapsed solid tumor patients and additional testing in Phase I and II clinical studies is ongoing (www.clinicaltrials.gov).

TNF family

CD27

CD27 is exclusively expressed by activated T cells, B cells and NK cells. Its ligand, CD70, is transiently up-regulated on T cells and B cells after antigen receptor stimulation and has also been found on thymic DC (63). CD27-CD70 ligation promotes TCR-induced expansion of CTL and T helper cells as well as plasma cell differentiation and antibody production in B cells (64,65). In addition, it is thought to protect against activation-induced cell death (AICD) (66). CD27- and CD70-specific antibodies are currently under clinical evaluation for the treatment of $CD27^+$ and $CD70^+$ tumors, respectively (www.clinicaltrials.gov). Furthermore, pre-clinical models are evaluating the generation of genetically modified tumor antigen-specific T cells (CARs). These CARs directly link the CD27 domain intracellularly which enables CD27-mediated co-stimulation (67).

CD30

CD30 was initially described as an antigen expressed on Hodgkin lymphoma cells (68). More recent studies reported CD30 expression in a small subset of activated T cells, B cells and eosinophils (69). CD30 promotes T cell proliferation in the

presence of TCR stimulation (70) and stimulates T cell-mediated production of IL-2, TNFα, IFNγ and IL-5 (71,72). In B cells, CD30 stimulates proliferation and antibody production (73,74). Its restricted expression on normal cells and high expression on lymphoma cells makes the CD30 receptor an ideal candidate for immunotherapy. Clinical evaluation of a CD30-specific antibody in patients with relapsed or refractory $CD30^+$ hematological malignancies showed an overall response rate of 50% and a complete response rate of 34% at a 3-week treatment interval (75).

OX40 (CD134)

OX40 is predominantly expressed on activated CTL and T helper cells, including Treg (76,77), while OX40 Ligand (OX40L) can be induced on activated B cells (78), DC (79), Langerhans cells (80), macrophages (81), NK cells (82), mast cells (83), vascular endothelial cells (84) and smooth muscle cells (85). OX40 promotes T cell division and survival (86) and protects against apoptosis (87). In addition, OX40 is shown to stimulate effector T cells to produce IL-2 (77) and to block the up-regulation of CTLA-4 (88), Foxp3 (89) and IL-10 (90) which augments ongoing immune responses. OX40-mediated immunotherapy is geared towards its immune-stimulatory properties and is postulated to enhance induced antitumor immune responses. Therefore, OX40-based immunotherapeutics will likely be employed as adjuvants and find a role in the combined use of various immunotherapeutic strategies. Patients suffering from metastatic prostate cancer are currently treated with an anti-OX40 antibody in a phase I clinical study which evaluates the combined use of anti-OX40, cyclophosphamide and radiation (www.clinicaltrials.gov).

4-1BB (CD137)

4-1BB (hereafter called CD137) is recognized as an important target for the treatment of both cancer and autoimmunity (91-95). Its expression is induced on both activated human T cells, NK cells and B cells (96-98), while the ligand (CD137L) is expressed on monocytes, DC, B cells and NKT cells (99-101). CD137-CD137L ligation results in T cell proliferation and increased cell survival (102) as well as NK-dependent antitumor immunity (92,94,100). Furthermore, unlike other co-stimulatory-based immunotherapies (e.g. CTLA-4 blockade), CD137 ligation does not result in self reactivity, but rather has therapeutic benefit in several murine models of autoimmune disease such as inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus (103-105). Although the mechanisms of CD137-mediated antitumor immunity and reduction of autoimmunity appear distinct, manipulation of this pathway is attractive for the

treatment of disease. First in-human studies of an agonistic fully humanized IgG4 monoclonal anti-CD137 antibody demonstrated promising clinical efficacy (106) and several clinical phase I and II studies in patients with various solid malignancies are ongoing. However, in some patients anti-CD137 antibody treatment was associated with severe liver toxicity. This side effect was seemingly dose-dependent and self-limiting in most cases, yet remains a major concern (107).

CD40

CD40 is constitutively expressed on B cells, macrophages, DC and platelets and can be inducibly expressed by endothelial cells (108). Binding to its ligand, CD40 Ligand (CD40L), which is primarily expressed on T cells (109), induces B cell proliferation and antibody production (110) as well as the activation and maturation of DC (19). CD40-mediated co-stimulation augments T cell-dependent antitumor immunity (17,19) while blocking the CD40 pathway is shown to prevent graft-versus-host disease (111). However, clinical trials testing anti-CD40 blocking antibodies were cancelled due to thromboembolic complications (112). In contrast, clinical testing of an agonistic anti-CD40 antibody in patients with advanced solid tumors was well tolerated and associated with objective tumor responses (36).

Table 2 Receptors and ligands belonging to the TNF family

receptor	expression	ligand	expression	signal	ref
CD27	T cells, B cells; NK cells	CD70	T cells; B cells; DC	activation	57
CD30	T cells; B cells; eosinophils	CD153	T cells; B cells	activation; inhibition	163
OX40 (CD134)	T cells	OX40L (CD134L)	B cells; NK cells; DC; macrophages; Langerhans cells; mast cells; smooth muscle cells	activation	164
4-1BB (CD137)	T cells; B cells; NK cells	CD137L (4-1BBL)	B cells; NKT cells; DC; monocytes	activation; inhibition	93, 96
CD40	B cells; DC; macrophages; platelets; endothelial cells	CD40L (CD154)	T cells	activation	165
GITR	T cells; B cells; NK cells; DC; macrophages	GITRL	B cells; DC; macrophages	activation	166
HVEM	T cells; APC	LIGHT	T cells; tumor cells	activation	167, 168

Ref indicates reference; DC, dendritic cell; NK cell, natural killer cell; NKT cell; natural killer-like T cell; GITR, glucocorticoid-induced TNF receptor; GITL, glucocorticoid-induced TNF receptor ligand; HVEM, herpesvirus-entry mediator; APC, antigen-presenting cell

Moreover, treatment of patients with surgically incurable pancreatic cancer with agonistic anti-CD40 antibody in combination with gemcitabine chemotherapy induced tumor regression in a subset of patients (113). Additional animal studies revealed that this tumor regression required macrophages, which facilitated the depletion of the tumor stroma. Further clinical testing of agonistic anti-CD40 antibodies is ongoing (www.clinicaltrials.gov) and additional treatment strategies in which tumor cells are modified to express CD40L are explored (114).

GITR

GITR is expressed on T cells, including Treg, activated B cells, macrophages, DC and NK cells (115,116). In contrast, GITR Ligand (GITRL) is expressed on DC, macrophages and B cells (117,118). Receptor-ligand interaction enhances T cell proliferation and the production of cytokines in an antigen-dependent manner. In addition, murine models provided evidence that GITR-expressing Treg lose their immunosuppressive function after GITR stimulation (119), although these findings seemed not reproducible in human *in vitro* models (120).

Current concepts in cancer immunotherapy

Immunotherapeutic strategies are divided into two major groups: (i) passive immunotherapeutic strategies directly targeting tumor cells or host-defending immune cells including monoclonal antibodies and (ii) active immunotherapeutic strategies inducing tumor-specific T cells and long-lasting antitumor immune responses including cytokine treatment, the adoptive transfer of T cells, NK cells or DC and vaccination with tumor-specific peptides, tumor-specific proteins, irradiated tumor cells, tumor-cell lysates or DC. To date, a total of eight immunotherapeutics have been approved for the treatment of solid tumors which are summarized in table 3.

Monoclonal antibodies

Monoclonal antibodies have been approved for the treatment of solid malignancies since 1998 (121). Their antitumor effect is partially dictated by the amount of antibody-specific antigen expressed on the target cell (122) and relies on (i) the direct delivery of apoptotic signals to the target cell, (ii) the direct inhibition of cancer-promoting signaling pathways, (iii) ADCC, (iv) complement-dependent cytotoxicity (CDC) and (v) the induction of antagonistic effects and agonistic effects on inhibitory and activating receptors, respectively. Currently, five monoclonal antibodies (Herceptin®, Erbitux®, Avastin®, Vectibix® and

Yervoy®) are approved for the treatment of solid malignancies and several others are under clinical evaluation (www.clinicaltrials.gov).

Cytokines

Cytokines are immunomodulating molecules which are critically important in the development of robust self-limiting immune responses including antitumor responses. Their secretion by various cells, including immune cells, facilitates immune signaling without the need of direct cell-to-cell interactions (123). In general, cytokines act as a growth factor and induce immune cell activation and proliferation, although they can also stimulate immune inhibition. In addition, they may enhance or suppress the production of other cytokines or exhibit antagonistic, additive or synergistic effects on overall immunity. To date, Interleukin-2 (IL-2) is approved for the treatment of metastatic renal carcinoma and melanoma, whereas Interferon-alpha (IFN-α) is approved for the adjuvant treatment of stage III melanoma and the combined use with Bevacizumab (Avastin®) in renal carcinoma. In addition, various cytokines are currently under clinical evaluation including IL-7, IL-12, IL-15 and IL-21 (www.clinicaltrials.gov).

Cytokines are highly pleiotropic in nature and thus, drive multiple immune-specific interactions (124). This is reflected by regularly observed, sometimes severe, side-effects in association with IL-2 or IFN-α therapy. Specifically, over 80% of treated cancer patients experience immune-related side effects including fever, fatigue, headache, gastrointestinal symptoms, myalgias, thrombocytopenia, leucopenia, neutropenia and increased hepatic enzymes under IFN-α therapy (125). Furthermore, depression (45%), confusion (10%), mania (<1%) and suicides (rarely) are reported (126,127). IL-2 therapy is associated with fever, chills, fatigue, gastrointestinal symptoms, transaminase elevation and cholestasis. In addition, IL-2 therapy may induce a capillary leak syndrome illustrated by hypotension, tachycardia, peripheral edema, cardiac arrhythmias and death (128-130). However, IL-2 therapy induces objective antitumor responses in 15% of patients with long-lasting antitumor responses in 5% of treated patients (128).

Cancer vaccines

Effective cancer immunotherapy is the net result of circulating tumor-specific T and B cells. Therefore, cancer vaccine strategies aim at the direct presentation of tumor-specific antigens to T and B cells. Initial cancer vaccine approaches facilitated tumor-antigen presentation by direct injection of tumor-specific peptides (131), tumor-specific proteins, tumor-specific long peptides (including Trojan peptide-based vaccines) (132,133), irradiated tumor cells or tumor-cell lysates.

Table 3 Approved immunotherapies for the treatment of solid tumors

name	type of treatment	specificity	type of cancer	approval	ref
Trastuzumab (Herceptin®)	antibody	Her2/neu	breast	1998	169
			gastric	2010	170
Cetuximab (Erbitux®)	antibody	EGFR	colorectal	2004	171
			SCCHN	2006	172
Bevacizumab (Avastin®)	antibody	VEGF	colorectal	2004	173
			NSCLC	2008	174
			glioblastoma	2009	175
			renal[a]	2009	176
Panitumumab (Vectibix®)	antibody	EGFR	colorectal	2006	177
Ipilimumab (Yervoy®)	antibody	CTLA-4	melanoma	2011	42
IL-2 (Proleukin®)	cytokine	n/a	renal	1992	178
			melanoma	1998	117
INFα (IntronA; Roferon®)	cytokine	n/a	renal[a]	2009	179
			melanoma	1996	180
Sipuleucel-T (Provenge®)	cellular therapy	n/a	prostate	2010	125

SCCHN indicates squamous cell carcinoma of the head and neck; NSCLC, non-small cell lung cancer; IL, Interleukin; IFN, Interferon; HER2/neu, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; VEGR, vascular endothelial growth factor; CTLA-4, cytotoxic T-lymphocyte antigen 4
[a]only in combination with Interferon-alpha

To enhance antigen-presentation and co-stimulation, cancer vaccine approaches were extended towards cellular vaccination with a primary focus on DC loaded or transfected with tumor-specific antigens. In this case tumor-specific antigens are presented in context of HLA class I and II molecules in the presence of co-stimulatory molecules expressed on the DC surface which have the potential to regulate the antitumor immune responses (134,135). Importantly, based on this premise, Sipuleucel-T (Provenge®) was shown to prolong overall survival with 4.1 months in a phase III trial conducted in patients with metastatic prostate carcinoma and resulted in the FDA approval of Provenge® as first cell-based immunotherapeutic strategy for the treatment of solid malignancies (136).

Adoptive cellular therapy

Adoptive cellular therapy (ACT) is a form of immunotherapy that is based on the *ex vivo* selection, activation, expansion and subsequent reinfusion of tumor-specific T cells into patients (137). To improve clinical responses, patients may be depleted

of immunosuppressive cell subsets prior to infusion. Objective tumor responses were observed in 56% of patients who received *ex vivo* expanded tumor-specific T cells along with IL-2 following a lymphodepletion protocol. In addition, 19 out of 20 patients experienced long-lasting and complete tumor regression with ongoing remissions beyond 3 years (138). Unfortunately, in some of the patients, tumor resection for isolation of tumor-specific T cells is not feasible. Furthermore, tumor-specific T cells fail to expand *ex vivo* in half of the cases assessed (137). Therefore, the genetic modification of autologous T cells with genes encoding for tumor-specific T cell receptors is explored. Early clinical results demonstrated complete tumor regression in two out of 17 metastatic melanoma patients (139) and further clinical testing is ongoing (www.clinicaltrials.gov).

Some studies focused on the *ex vivo* expansion and subsequent adoptive transfer of NK cells in patients with solid malignancies (140,141), since NK cells were shown to mediate direct tumor cell killing (142). In addition, NK cells are thought to induce DC maturation and to facilitate cross-presentation of tumor-specific antigens derived from killed tumor cells (143). Some clinical benefit has been reported in patients with advanced non-small cell lung cancer (NSCLC) (144) and additional studies are ongoing

Obstacles to successful application of immunotherapeutic strategies

Successful cancer immunotherapy does not solely depend on the effective generation of high numbers of tumor-specific immune cells. These cells must localize the tumor and infiltrate the tumor microenvironment in order to clear primary or metastatic disease. Unfortunately, tumor cells can escape from antitumor immunity through a variety of mechanisms. In addition, successful cancer immunotherapy is further affected by the wide diversity of immune deficiencies found in individual cancer patients. Mechanisms associated with impaired antitumor immunity include (i) tolerance of T cells to tumor-specific antigens, (ii) active suppression of tumor-specific T cells, (iii) defects in professional antigen-presenting cells (APC), (iv) malfunctioning T cells as a consequence of alterations in their TCR and (v) loss of HLA class I antigens.

Immune tolerance

Immune tolerance is mediated, in part, by both the induction of T cell anergy and deletion of antigen-specific T cells from the peripheral circulation. As described previously, T cell anergy occurs when TCRs recognize antigen in the absence of

proper co-stimulation (145), which results in the failure of antigen-specific T cells to produce IL-2 and clonally expand upon antigen exposure (146). In addition, tumor antigen-specific T cells may directly be deleted from the peripheral circulation through AICD which is characterized by apoptosis of activated T cells upon antigen encounter (147). Fas and FasLigand (FasL) mediated mechanisms also play a role in the deletion of T cells. FasL is expressed in various types of human malignancies (148) and it has been shown that T cell apoptosis is abrogated by anti-FasL antibodies or Fas fusion protein (149). This form of T cell deletion is, in general, tumor-antigen independent.

Immunosuppresion within the tumor microenvironment

In the tumor microenvironment, suppression of T cells is mediated in various ways, including the expression of PD-L1 and galectine-1 on the tumor cell surface. As described previously, blocking of PD-1/PD-L1 interactions results in improved antitumor T cell functions both *in vitro* and *in vivo* (150,151). In addition, the expression of tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) (152) and the secretion of soluble factors like TGF-β (153) and IL-10 (154) lead to T cell dysfunction and apoptosis. Furthermore, several tumor infiltrating cell subsets actively suppress antitumor T cells. Among these tumor infiltrating cell subsets are Treg (155), which suppress both T cell proliferation and NK cell mediated cytotoxicity (155,156) and myeloid derived suppressor cells (MDSC). The latter cell population strongly inhibits T cell function through the expression of arginase and inducible oxide synthase (iNOS) (157). In addition, in the presence of tumor, T cells have shown to develop a senescent-like phenotype characterized by the loss of CD28 and CD27. These tumor-induced senescent T cells mediate T cell suppression through direct cell-to-cell interactions (158).

Deficiencies in APC and TCR abnormalities

Deficiencies in APC include (i) decreased numbers of mature DC in the tumor micro-environment, draining lymph node and peripheral circulation, (ii) phenotypic alterations as demonstrated by low HLA class II and co-stimulatory molecule expression and (iii) a decreased ability to present soluble antigen to autologous T cells (159-161). In patients with advanced cancer stages, abnormalities of the CD3/TCR complex (TCRζ) and signal transduction pathways cause T cell dysfunction through impaired tyrosine phosphorylation of the CD3/TCR complex (162).

Downregulation of HLA class I molecules

It is extensively documented that solid tumors are able to evade the immune system through down-regulation or loss of HLA class I molecules (163) and abnormalities in class I molecules play a negative role in the clinical manifestation of cancer. Likewise, it is reported that the expression level of tumor associated peptide/HLA class I complexes is associated with recognition of tumor by CTL (164). For both cases it is shown that the addition of IFNγ restores and/or enhances the expression of HLA surface molecules as well as tumor associated peptide/HLA class I complexes *in vitro* (165) but this is yet to be demonstrated *in vivo* in humans.

Tumor-antigen selection

The ideal tumor-antigen is highly expressed on the cell surface of cancer cells and has a high binding affinity for HLA class I and/or class II molecules. Preferably, it is expressed by most cells within a particular tumor, and required to maintain the tumor phenotype. It should not be expressed in the thymus or during fetal development to minimize tolerance.

To date, no tumor-antigen naturally satisfies all the criteria to be classified as "ideal" cancer immunotherapeutic candidate. Most antigens (e.g. epitopes) are identified by mass spectrometry (166), which can help identify tumor-antigens but does not provide any information on the immunologic potential of these antigens. Selection is further hindered by that fact that many antigens undergo post-translational modifications (PTMs). These modifications directly affect the stability and function of proteins and naturally processed antigen epitopes (167) and thus, affect the ability of antigen specific T-cells to recognize the modified epitope. The screening of PTMs in naturally processed peptides is technically challenging and therefore has major clinical implications.

In summary, it is clear that individual cancer patients exhibit a variety of mechanisms which compromise effective antitumor immunity. Cancer patients likely possess several alterations in their antitumor immunity and therefore require immunotherapeutic strategies which are able to induce antitumor immunity and at the same time target these immune deficiencies. Cancer patients are likely to require individually chosen immunotherapeutic strategies or a combination of various treatment modalities, rather than a single treatment modality at a time.

Outline of the thesis

Increasing clinical data suggest that eradication of human cancer may best be accomplished by concurrent activation of the various components of the immune system. This may be achieved by combining cancer treatment modalities which are able to enhance antitumor immune responses within the innate or adaptive immune system, by combing cancer treatment modalities, which simultaneously activate both innate and adaptive immunity or by combining treatment modalities, which stimulate tumor-specific immunity and target co-stimulatory pathways. At first, most treatment strategies prioritized the stimulation of CTL responses based on their ability to directly and specifically kill tumor cells. Currently, most immunotherapeutic strategies in patients with solid tumors are geared towards direct elimination of tumor cells or direct activation of the adaptive immune system, whereas relatively few are intended to stimulate co-signaling pathways or the innate immune system. For that reason, this thesis focuses on CD137-mediated co-stimulation **(part A)** and NK cell-based immunotherapy **(part B)** and their therapeutic potential in the treatment of solid malignancies. In addition, results of a clinical pilot study testing a MAGE and HPV peptide-based vaccine, which was shown to stimulate both CTL and T helper cell responses in murine models are presented and discussed **(part C)**.

Specifically, preclinical studies of a glycosylated and an aglycosylated anti-CD137 antibody are reported since stimulation of CD137, through its natural ligand or agonistic antibody, has been shown to induce antitumor immunity in murine models. Based on the crucial role of murine NK cells in CD137-mediated antitumor immunity, comprehensive *in vitro* studies of the direct effect of the glycosylated and aglycosylated chimeric CD137 antibodies on human NK cells were performed. In addition, considering the role of B cells in antitumor immune regulation, the co-stimulatory function of CD137 on human B cells was elucidated. These studies are reported in **chapter 2 and 3**, respectively. In addition, to facilitate clinical translation of the CD137 mAbs, *in vitro* non-human primate studies were conducted to ascertain feasibility and relevance of preclinical animal testing. These findings, reported in **chapter 4**, underscore the fundamental importance of preclinical *in vitro* animal model evaluation for *in vivo* relevance, since humanized anti-CD137 did not bind CD137-expressing macaque or baboon peripheral blood mononuclear cells (PBMC). This was directed by a three amino acid difference within the extracellular domain of human CD137 between macaque and baboon CD137.

Based on (i) the pivotal role of NK cells in CD137-mediated antitumor immunity, (ii) the reported success of NK cell-based immunotherapy in patients affected with hematological malignancies and (iii) the importance of CD137-CD137L ligation in *ex-vivo* NK cell expansion additional studies focused on NK cell-mediated antitumor cytotoxicity as a means to potentially boost innate immunity. In **chapter 5**, the successful *ex-vivo* expansion of GMP-compliant NK cells from patients with solid malignancies is reported. Expansion turned the NK receptor balance towards activation and resulted in cytotoxicity against autologous gastric tumor cells. In addition, cytotoxic activity could be enhanced by ADCC. Furthermore, in **chapter 6**, an *in vitro* head and neck cancer model provides first preclinical evidence of the importance of FcγRIII polymorphisms in NK cell-mediated ADCC in solid malignancies.

Finally, in the clinical study described in **chapter 7**, we were able to cross the line between scientific possibility and clinical reality. This chapter reports first in-human data of the clinical evaluation of two peptide-based vaccines in patients suffering from head and neck carcinoma. It describes the successful *in vivo* induction of vaccine-specific T cell responses and in addition, comments on unexpected difficulties and how they were resolved. In **chapter 8**, all results and conclusions are summarized and discussed.

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PART A

CD137 mediated co-stimulation, its therapeutic potential and challenges in clinical translation

2

Fc dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies

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Abstract

CD137 (4-1BB) is a co-stimulatory molecule which can be manipulated for the treatment of cancer and autoimmune disease. While agonistic antibodies (mAbs) against CD137 enhance the rejection of murine tumors in a natural killer (NK) and T cell dependent fashion, the mechanism for NK dependence is poorly understood.

In this study, we evaluated the ability of two different glycoforms of a chimerized anti-human CD137 mAb, an aglycosylated (GA) and a low fucose form (GG), to react with human NK cells. Both mAbs bound similarly to CD137, were competitively inhibited by the parental mAb and partially blocked the interaction between CD137 and CD137Ligand. However, unlike GA mAb, immobilized GG mAb activated NK cells and enhanced CD137 expression. These effects were seemingly dependent on Fc interaction with putative Fc-receptors on the NK cell surface, as only the immobilized Fc-fragment of GG was required for CD137 expression. Furthermore, CD137 expression could be enhanced with antibodies directed against non-CD137 epitopes, and the expression levels directly correlated with patterns of Fc-glycosylation recognized to improve Fc interaction with Fcγ- receptors.

Our data suggest that CD137 can be enhanced on NK cells in an Fc dependent fashion and that expression correlates with phenotypic and functional parameters of activation.

Introduction

CD137 (4-1BB), a member of the TNF receptor superfamily, is increasingly recognized as an important target for the treatment of both cancer and autoimmunity (1-5). Specifically, in murine models, it is clear that ligation of CD137 on the surface of activated T cells, through either CD137 ligand (CD137L) or agonistic monoclonal antibodies (mAbs), potentiates the immune response to weakly immunogenic tumors in a NK dependent fashion (3-6). Unlike other co-stimulatory based antitumor immunotherapies (e.g. CTLA-4 blockade), CD137 ligation does not induce self reactivity, but rather has therapeutic benefit in multiple murine models of autoimmune disease such as rheumatoid arthritis (7), systemic lupus erythematosus (8), and inflammatory bowel disease (9). In many studies, functional conclusions regarding CD137 have been based on mAb or fusion protein manipulation of receptor/ligand pathways, with the assumption being that observed effects were secondary to Fab region or ligand interaction with CD137. Importantly, little attention has been paid to the potential link between the Fc region of these molecules and alternate pathways of CD137 regulation through Fc receptors (FcγRs).

It is now evident that Fc cross-linking of FcγRIII (CD16) on human NK cells induces cellular activation defined by both phenotypic change and release of pro-inflammatory cytokines (10). The affinity and functional consequences of the interaction between Fc and FcγRIII is dependent on the presence of oligosaccharides (N-glycan) covalently attached at asparagine (Asn)-297 of the Fc heavy chain (11). For example, Fc fragments with low fucose content at Asn-297 have enhanced binding affinity for FcγRIII (12-14). Additionally, aglycosylated Fc fragments are unable to efficiently bind the FcγRIII (15,16). The interaction between Fc-FcγRs also has clinical implications, as it is now evident that polymorphisms within the FcγRIII, e.g. V/F at amino-acid position 158, which impact Fc-FcγRIII interactions, can be used to define the therapeutic efficacy of targeted anti-cancer therapeutics such as Rituxan® (17,18).

Based on the therapeutic potential of anti-CD137 mAbs and the recognized importance of Fc-FcγR interactions on mAb function, in collaboration with GTC Biotherapeutics, two chimeric anti-CD137 mAbs were developed. The first, a glycosylated form (GG) is likely to crosslink the FcγR and the second, an aglycosylated form (GA), is unlikely to efficiently crosslink the FcγR. Because of the recognized role of NK cells in the antitumor function of anti-CD137 mAbs in murine models, we initially hypothesized that IL-2 stimulated human NK cells

would express CD137 and that ligation with chimeric anti-CD137 mAb would result in cytokine release and degranulation.

Surprisingly, we observed that, following IL-2 stimulation and subsequent culture, human NK cells do not express high levels of CD137. However, CD137 is enhanced on IL-2 stimulated human NK cells following culture in the presence of immobilized glycosylated mAbs or papain cleaved Fc fragments. The ability to enhance CD137 expression is independent of the antigen specificity of the Fab region and the magnitude of CD137 expression is associated with patterns of glycosylation known to enhance the interaction between Fc and FcγRs. These data suggest that "agonistic effects" of select antibodies on co-stimulatory molecules, may be in part secondary to Fc-FcγR interactions and provide important insight into the design of antibodies intended to manipulate co-stimulatory pathways for clinical benefit.

Materials and Methods

Chimeric monoclonal antibodies

Mouse anti-human CD137 mAb (G6) was generated in the laboratory of Dr. L. Chen. A glycosylated chimeric version of G6, hereafter called GG, was developed by replacing the mouse IgG2a Fc region with a human IgG1 Fc region. Likewise, an aglycosylated (GA) chimeric anti-CD137 mAb was created by mutating Asn to glutamine (Gln) at amino-acid position 297 in the Fc region. Both GA and GG mAbs were produced in the milk of transgenic goats. Erbitux® (cetuximab, hereafter called CTM) was obtained through the Greenebaum Cancer Center (University of Maryland Medical System, Baltimore, MD). For flow cytometry experiments, all chimeric mAbs were directly labeled to Allophycocyanin (APC) through the custom conjugation service of Invitrogen (Molecular Probes Invitrogen, Eugene, OR).

Cells and transfectants

Human blood samples were purchased (Biological Specialty Corporation, Colmar, PA) and whole peripheral blood mononuclear cells (PBMC) were isolated using density-gradient centrifugation. Fresh purified resting NK cells ($CD3^-CD56^+CD16^+$ as defined by flow cytometry) were obtained by negative magnetic cell sorting using NK cell isolation beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Chinese hamster ovary (CHO) cells stably transfected with human CD137 receptor and non-transfected wild type controls were established in the laboratory of Dr. L. Chen. CHO cells were maintained in DMEM

supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological, Lawrenceville, GA), 1% penicillin/streptomycin (P/S), 1% Glutamax-1 (both Invitrogen, Grand Island, NY) and 1% HEPES (Mediatech, Herndon, VA). K562 cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% P/S and 1% Glutamax-1.

In vitro NK experiments

Fresh purified resting NK cells ($CD3^-CD56^+CD16^+$ as defined by flow cytometry; >90% purity) were stimulated for 72 hours in RPMI 1640 supplemented with 10% FBS, 1% P/S, 1% HEPES, 1% Glutamax-1 and 200IU/ml recombinant human IL-2 (rhIL-2, Proleukin®, Chiron Corporation, Emeryville, CA). After 72 hours, NK cells were washed three times with PBS and resuspended in RPMI 1640 supplemented with all of the above except rhIL-2. IL-2 stimulated NK cells were plated at a concentration of $2x10^5$ cells/well in a 96-well flat bottom plate in the presence of either pre-coated (immobilized) or soluble chimeric mAb or polyclonal human IgG1 (huIgG1) (Sigma-Aldrich, St. Louis, MO) at a concentration of 20μg/ml. Control wells were pre-coated overnight with RPMI 1640 supplemented with 10% FBS to minimize cross-linking of soluble positively charged immunoglobulins present in the culture media. At the indicated time-points, NK cells and cell-free supernatants were harvested and analyzed for cell surface expression of defined proteins and secreted cytokines.

Flow Cytometry

NK cell phenotype was determined by staining with directly conjugated mouse anti-human mAbs against CD3, CD56, CD16, CD69, CD54, and CD137 (BD Biosciences, San Diego, CA). Unless indicated otherwise, CD137 expression was measured using commercial mouse anti-human CD137 mAb (clone 4B4-1). In some experiments, NK cells were stained with Annexin V/7-AAD (Annexin V apoptosis detection kit I, BD Biosiences) to distinguish live cells from apoptotic and/or necrotic cells. The number of degranulating NK cells was determined by staining with CD107a mAb (BD Biosciences). In brief, NK cell cultures were incubated for one hour at 37°C in 5% CO_2 after which brefeldin A and monensin (both BD Biosiences) were added. NK cells were cultured for an additional five hours and subsequently stained for CD56 and CD16. Directly conjugated mouse IgGs were used as isotype controls. A minimum of 10000 events were acquired using a BD™ LSR II flow cytometer and analyzed with BD™ FACS DIVA Software.

Analysis of collected cell-free supernatants

Cell-free culture supernatants were collected at indicated time-points during incubation. Concentrations of IFN-γ and TNF-α were determined by ELISA according to the manufacturer's instructions (BD Biosciences).

Flow Cytometry based competitive binding assays

CD137 expressing CHO cells were incubated with different concentrations (0.8μg/ml-100μg/ml) of G6, GA and GG mAb at 4˚C for 1hr. Purified mouse IgG2a (muIgG2a) and huIgG1 (BD Biosciences) were used as control antibodies at a concentration of 100 μg/ml. Next, cells were incubated with 5ug/ml of human CD137L-muCD8 (Ancell, Bayport, MN) for 45 minutes at 4˚C. Cells were further stained with PE conjugated anti-muCD8 mAb (BD Biosiences) and acquired using a BD™ LSR II flow cytometer.

Generation of Fab fragments and Fc fragments

Fab and Fc fragments from GG, CTM and huIgG1 were generated by papain digestion followed by protein A and protein L chromatography using an ImmunoPure Fab Preparation kit (PIERCE, Rockford, IL) according to the manufacturer's instructions. Briefly, all mAbs were digested into Fc and Fab fragments using papain. The digests were dialyzed against protein A or protein L binding solutions, subsequently passed through protein A or protein L columns and the flow through, containing Fab or the Fc fragments respectively, were collected. Purity, determined by both immunoblotting and ELISA, was greater than 75% and 95% for the Fc and Fab fractions respectively.

Compositional monosaccharide analysis of IgG1-Fc

Purified Fc fragments (100-200μg) were diluted to 200μl using deionized water and mixed with an equal volume of 4N TFA (trifluoroacetic acid, Sigma Aldrich, St.Louis, MO). Samples were hydrolyzed at 100°C for 5h. After lyophilization, the residues were reconstituted with water prior to HPAEC analysis. The chromatography was performed on a Dionex DX600 chromatography system (Dionex Corporation, Sunnyvale, CA) equipped with an electrochemical detector (ED50). Monosaccharides were separated on a CarboPac PA10 column (4X250 mm) with an isocratic 18 mM NaOH as the eluent. Monosaccharides were detected by the pulsed amperometric electrochemical detector with the following waveforms (potentials and durations): E1 = +0.05 V (T1 = 0 to 04 s), E2 = +0.75 V (T2 = 0.41 to 0.6 s), and E3 = -0.15 V (T3 = 0.61 to 1 s). Typically, 10μl of sample containing 0.1-1nmole of monosaccharides was injected. For quantification, the

peaks corresponding to the monosaccharides were integrated and peak areas were normalized to molar quantity by comparison with standard solution of monosaccharide standards.

Cytotoxicity assays

NK cytotoxicity was measured by 4 hour chromium 51 (^{51}Cr)-release assays. K562 tumor targets were labeled with 150 µCi ^{51}Cr ($Na_2{}^{51}CrO_4$, Perkin Elmer, Shelton, CT) for one hour at 37°C in 5% CO_2. After incubation, cells were washed two times and incubated for an additional 30 minutes in order to reduce spontaneous release. Finally, tumor targets were plated at 5000 targets/well in triplicate wells in a 96-well V-bottom plate. Effector cells consisted of $CD3^-CD56^+$ NK cells which were IL-2 stimulated for 72 hours and subsequently cultured for 24 hours in the presence of immobilized GA or GG mAb. One hundred microliter of NK effectors were added at indicated effector:target ratio to give a final volume of 200µl per well. After 4 hours of incubation, 100µl of supernatant was harvested and mixed with 100µl of scintillation cocktail (Perkin Elmer) in a 96-well sample plate (Perkin Elmer). Its radioactive content was measured using a gamma-counter and the percentage of specific lysis was calculated using the formula: 100 X (experimental release – spontaneous release)/(maximum release – spontaneous release).

Statistical analysis

A Q-Q plot was used to assess if normality could be assumed for the flow cytometric parameters. Statistical significance, as measured by a two-sided paired Student's t test (or the non-parametric sign test if the normality was not plausible) was calculated using Excel v2003 and Splus, based on the number of experiments as indicated in the figure legends. NK cell cultures with immobilized GA mAb were used as control cultures to calculate statistical differences. Differences were considered to be significant at $P< 0.05$.

Results

Immobilized glycosylated mAbs enhance CD137 expression on human NK cells

Several groups, including ours, have demonstrated in murine models that the antitumor activity of anti-CD137 agonistic mAbs is NK dependent (2,3,6). Based on the crucial role of NK cells in antitumor activity and the recognized expression of CD137 on IL-2 stimulated murine NK cells, we initially hypothesized that human NK cells cultured in IL-2 would express high levels of CD137 and that

chimeric anti-CD137 mAbs would react with CD137 expressed on IL-2 stimulated human NK cells, inducing degranulation and cytokine release.

Interestingly, we found that following IL-2 stimulation and subsequent culture in RPMI 1640 supplemented with FBS, human NK cells, did not demonstrate high levels of CD137 expression (data not shown). In contrast, IL-2 stimulated NK cells cultured in the presence of immobilized glycosylated mAbs showed high levels of CD137 expression (Figure 1). Expression of CD137 was transient and was not observed to a measurable extent after 72 hours of culture (data not shown). Of note, NK cells from one individual evidenced relatively high levels of CD137 after IL-2 stimulation. This exception may reflect ongoing infection/inflammation in this donor or individual variation in susceptibility for IL-2.

CD137 was only enhanced on IL-2 stimulated NK cells in cultures with immobilized GG (range 22%-61%; P=0.0071) and not with GA (range 1%-14%). Cultures of IL-2 stimulated NK cells in the presence of non-immobilized (soluble) mAbs failed to induce CD137 expression. CD137 was also up-regulated on IL-2 stimulated NK cells cultured with immobilized polyclonal huIgG1 (range 3%-73%; P=0.0232) and, to a lesser extent with immobilized anti-EGFR mAb CTM (range 3%-10%). The Fab region of GA and GG did not appear to augment CD137 expression, as the levels of CD137 in cultures with immobilized huIgG1 + immobilized GA (range 14%-25%) were lower than in cultures with immobilized huIgG1 or immobilized GA alone (range 3%-73% and 1%-14% respectively).

Additionally, CD137 expression was inversely associated with the presence of FcγRIII. NK cell cultures with high levels of CD137 showed low levels of FcγRIII as indicated by CD16 in figure 1. Likewise, cultures with low levels of CD137 were associated with high levels of FcγRIII. To avoid possible interference of purified chimeric anti-CD137 mAbs in flow cytometry analysis, CD137 expression levels were determined using commercial mouse anti-human CD137 mAb (clone 4B4-1), whose binding to CD137 was not blocked by ten fold differences in concentration of GA or GG mAb (range 0.02μg/ml to 20μg/ml) as determined by flow cytometry based competition-inhibition studies (data not shown).

In order to exclude other possible sources of NK cell activation, we indirectly tested for the presence of LPS and evaluated the binding affinity of individual mAb preparations.

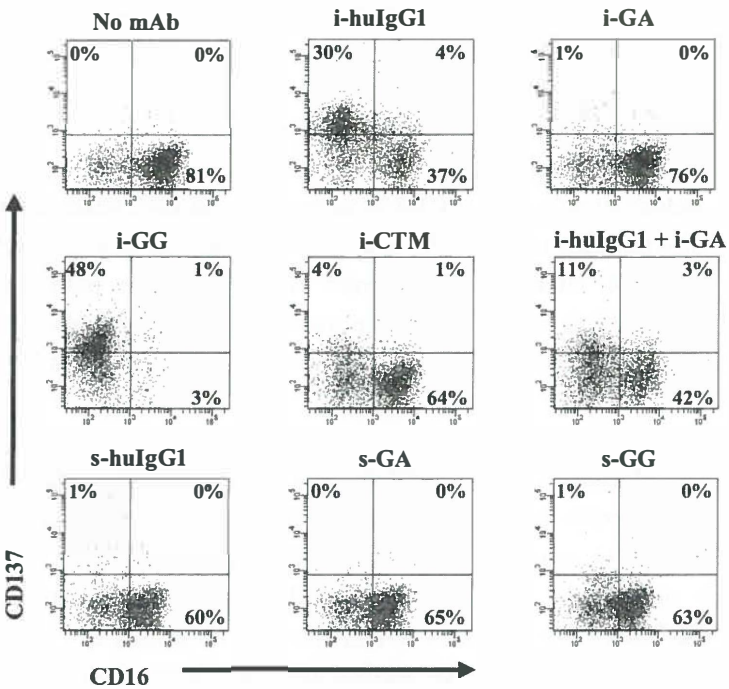

Figure 1. Immobilized glycosylated mAbs stimulate CD137 expression on human NK cells. IL-2 stimulated NK cells were cultured for 24 hours in the presence of immobilized or soluble chimeric mAbs as indicated. CD137 expression was measured by flow cytometry. Gates were set around $CD56^+$ (NK) cells and numbers in the dot plots indicate percentage of $CD56^+$ (NK) cells expressing CD137. Immobilized mAb culture conditions and soluble mAb culture conditions are indicated with i- and s- respectively. The data shown is representative of one of six individual experiments. Statistical analysis is based on six experiments; CD137 expression levels are significantly increased in NK cell cultures with i-huIgG1 or i-GG (P=0.0232 and P=0.0071, respectively) as compared with i-GA cultures. HuIgG1 indicates polyclonal human IgG1; GA, aglycosylated chimeric anti-CD137 mAb; GG, glycosylated chimeric anti-CD137 mAb; CTM, cetuximab.

The up-regulation of CD137 on the NK cell surface was likely not induced by LPS contamination, since boiling the GG mAb preparation eliminated its ability to up-regulate CD137 (data not shown). Furthermore, observed differences in CD137 expression were also not likely to be secondary to variable levels of immobilized immunoglobulins since similar levels of immobilized GG, GA and huIgG1 were present on the surface of pre-coated wells (data not shown). In addition, 10 fold increases in GG concentration did not further enhance CD137 expression on IL-2 stimulated NK cells. However, CD137 expression levels were slightly enhanced with a ten fold increase in immobilized CTM concentration (10μg/ml to 100μg/ml) and CD137 started to reach expression levels as observed in cultures with 10μg/ml GG (data not shown). These experiments support the conclusion that CD137

expression on NK cells is Fc dependent and that different Fc regions have variable ability to induce CD137 on human NK cells.

CD137 expression on NK cells is induced in an Fc dependent fashion

The fact that immobilized glycosylated mAbs, but not soluble glycosylated mAbs or aglycoslyated mAb, induce high levels of CD137 expression on IL-2 stimulated NK cells strongly supports the idea that CD137 expression on the NK cell surface is mediated through Fc-FcγR interactions. In order to exclude the potential influence of a CD137 specific Fab region in non-Fc mediated CD137 expression, purified Fab and Fc fragments of GG mAb were employed in IL-2 stimulated NK cell experiments.

As expected, NK cells cultured with immobilized GG/Fc fragments induced CD137 expression at levels similar to those observed with immobilized GG mAb (range 27%-61% and 22%-61%, respectively). In contrast, NK cells cultured with immobilized GA mAb or GG/Fab fragments only induced small amounts of CD137 expression (range 1%-14% and 6%-13%, respectively) as shown in Figure 2A-i and this difference was determined to be statistically significant (P=0.0002). Interestingly, the total number of live NK cells (gated as 7-AAD$^-$/AnnexinV$^-$ cells) decreased in the culture with immobilized GG mAb versus the culture with purified GG/Fc fragments as indicated by the increased ratio of total number of live cells versus total number of acquired cells, 1/6.7 and 1/3.7 respectively (Figure 2A-ii).

CD137 was not only detected by commercial mouse anti-CD137 mAb but also by both chimeric anti-CD137 mAbs, demonstrating that GG and GA cross-react with CD137 induced on IL-2 stimulated NK cells. These data have importance for future clinical translational studies (Figure 2B). Importantly, the purity of the Fab fragments was 95% and of the Fc fragments 75%. However, given that the Fab fragments did not induce high levels of CD137, it is unlikely that our results are secondary to contamination. Therefore, these data provide convincing evidence that CD137 is induced on IL-2 stimulated NK cells in an Fc dependent fashion.

Chimeric GA and GG mAb have similar Fab regions despite differences in Fc

In order to further evaluate the potential role of the Fab fragments of GA and GG in inducing CD137 on the NK cell surface, we compared their ability to bind 4-1BB transfected CHO cells and to block natural 4-1BB-4-1BBL interactions. Both mAbs showed similar binding to CHO/CD137 cells and failed to bind wild type (CHO/WT) controls (Figure 3A). To determine if the parent mAb (mouse anti-

human CD137) G6 and both chimeric mAbs GA and GG recognized the same CD137 epitope, CHO/CD137 cells were incubated with increasing concentrations of purified G6, GA or GG mAb respectively (Figure 3B). All mAbs partially blocked binding of G6-APC in a dose-dependent fashion, suggesting that chimerization did not alter epitope affinity.

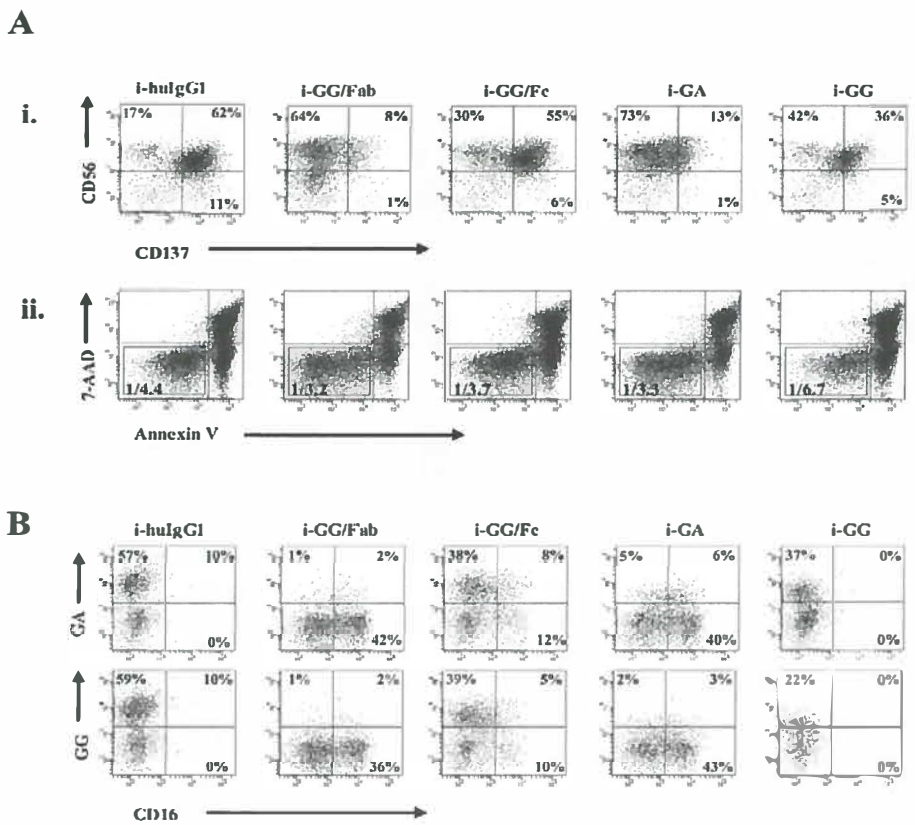

Figure 2. CD137 expression on NK cells is induced in an Fc dependent fashion. GG mAb was digested into separate Fab and Fc fragments using papain. Digests were passed over Protein L or Protein A columns and single Fab fragments or Fc fragments were collected respectively. IL-2 stimulated NK cells were cultured in the presence of immobilized chimeric mAb or immobilized fragments as indicated in the figure. **(A)** After 24 hours, CD137 expression was determined by flow cytometry. Gates were set around 7-AAD$^-$/AnnexinV$^-$ cells. Numbers in the dot plots indicate the percentage of CD56$^+$ (NK) cells expressing CD137 (i). Ratios represent the number of live NK cells and are calculated based upon the total number of 7-AAD$^-$/AnnexinV$^-$ cells within the total number of acquired cells (ii). **(B)** CD137 expressed on NK cells is not only recognized by commercial available mouse-anti-CD137 mAb but also by both chimeric anti-CD137 mAbs, GA and GG. Experiment shown represents one of three individual experiments. Statistical analysis is based on six (i-GG and i-GA) and three (i-GG/Fc and i-GG/Fab) experiments; CD137 expression levels are significantly increased in NK cell cultures with i-GG and i-GG/Fc as compared with i-GA and i-GG/Fab (P=0.0002). HuIgG1 indicates polyclonal human IgG1; GA, aglycosylated chimeric anti-CD137 mAb; GG, glycosylated chimeric anti-CD137 mAb.

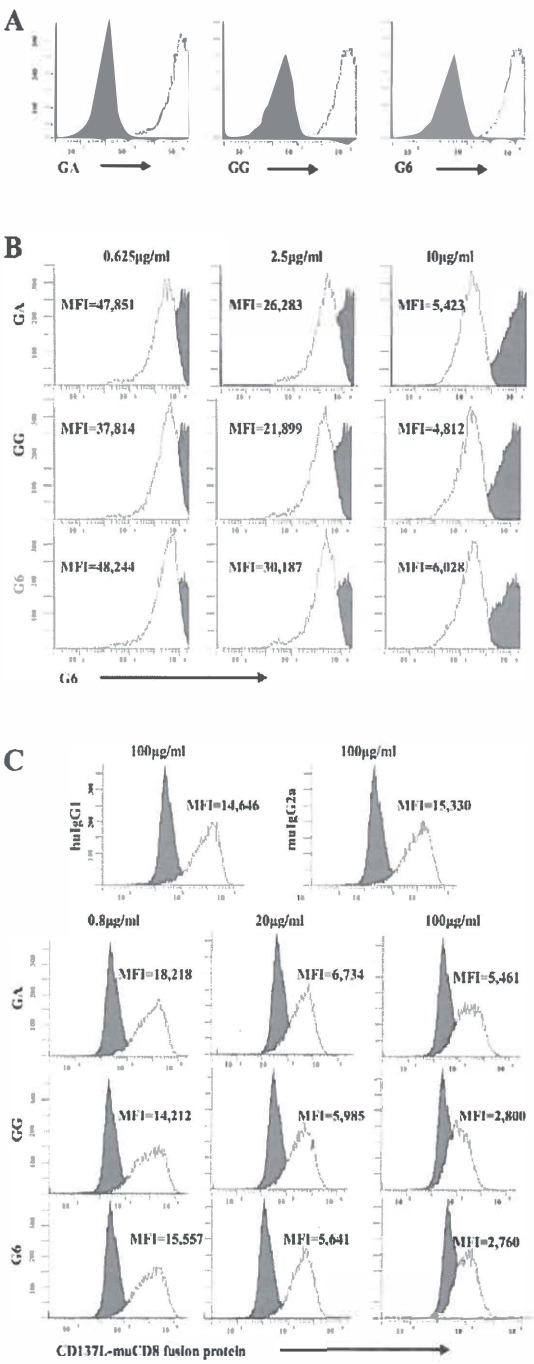

Figure 3 Chimeric GA and GG mAb have similar Fab regions despite differences in Fc. (A) G6, GA and GG mAbs bind similar to CD137 expressing CHO cells and fail to bind CHO/WT controls. **(B)** CD137 expressing CHO cells were incubated with different concentrations of purified G6, GA or GG mAb. Cells were subsequently stained with G6-APC mAb. Increasing concentrations of purified G6, GA or GG mAb blocked binding of G6-APC as indicated by MFI in a dose-dependent fashion. **(C)** CD137 expressing CHO cells were first incubated with increasing concentrations of G6, GA or GG and subsequently incubated with human CD137L-muCD8 fusion protein. Cells were then stained with PE conjugated anti-muCD8. As indicated by MFI, G6, GA and GG blocked CD137/CD137L interaction in a dose-dependent fashion. Filled histograms represent CHO/WT cells (A) or isotype control mAb (B+C). Open histograms represent staining with the indicated antibody. GA indicates aglycosylated chimeric anti-CD137 mAb; GG, glycosylated chimeric anti-CD137 mAb; G6, mouse anti-human CD137 mAb; MFI, mean fluorescence intensity.

Finally, to further define the functional characteristics of these mAbs, the ability of G6, GA and GG to block the interaction of the CD137 receptor with its natural ligand, CD137L was determined. CHO/CD137 cells, incubated with increasing concentrations of purified G6, GA or GG, blocked the binding of human CD137L/mouse CD8 fusion protein in a dose-dependent fashion (Figure 3C). These data indicate that both chimeric mAbs bind similarly to CD137 and partially block CD137 receptor-ligand interactions in a dose-dependent fashion suggesting equal specificity of their Fab region for the CD137 receptor. Thus, the fundamental difference between GA and GG mAb is the status of N glycosylation of the mAb Fc region. The fact that the Fab fragments of these antibodies are identical suggests that differences in CD137 expression levels in NK cell cultures with immobilized GG and GA mAb are secondary to the variable ability of the Fc regions of GG and GA to interact with putative FcγRs expressed on human NK cells.

CD137 expression levels on NK cells are associated with defined patterns of Fc glycosylation

Human IgG1 mAbs carry a conserved N-glycan at Asn-297 of the Fc region. Fc-FcγR interactions are dependent on the extent of core fucosylation of the Fc N-glycan, with lower fucosylated IgG1 having significantly enhanced affinity for the FcγIII receptor in comparison to those with high fucose content. The level of fucosylation has functional implications as lower fucosylated mAbs induce more potent antibody-dependent cellular cytotoxicity (ADCC) at lower antigen densities (13). Based on our observations that mAbs from different sources stimulated variable levels of CD137 on the NK cell surface, we sought to investigate if the difference in fucose composition of N-glycans was associated with CD137 expression. In order to quantitate the monosaccharide composition, we performed HPAEC profile analysis of hydrolysates of the different mAbs studied. To exclude the interference of potential glycosylation at other sites, we purified respective IgG1-Fc fragments from all mAbs and analyzed the monosaccharide composition of the Fc N-glycan. Treatment of the mAb Fc fragments with trifluoroacetic acid resulted in complete hydrolysis of N-glycan into the compositional monosaccharides, where N-acetylglucosamine (GlcNAc) was released as glucosamine (GlcN). The HPAEC profiles of the hydrolysates of all IgG1-Fc are demonstrated in Figure 4. As expected, the hydrolysis of GA did not release typical monosaccharides, e.g.. Mannose (Man), GlcN, and Galactose (Gal) of the N-glycan, confirming that no N-glycan was attached to this antibody. In contrast, GG, CTM and huIgG1 revealed typical monosaccharide patterns as compared with a standard monosaccharide profile.

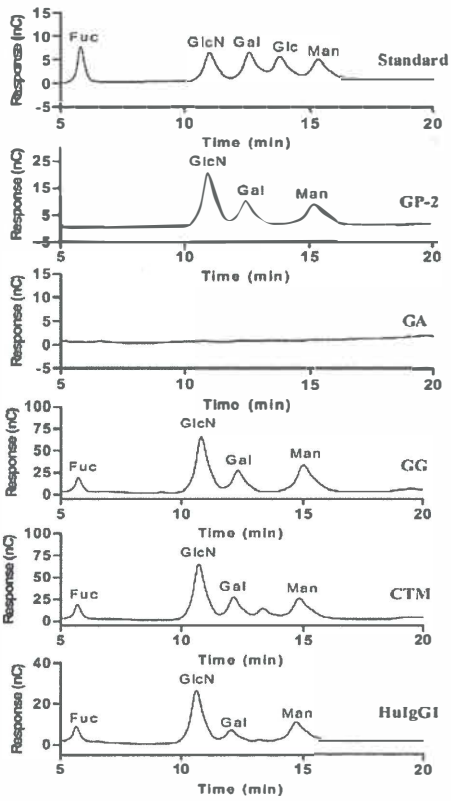

Figure 4 HPAEC profile analysis of IgG1-Fc. Trifluoroacetic (TFA) hydrolysis resulted in complete degradation of the N-glycan within the separate Fc fragments to compositional monosaccharides where N-acetylglucosamine (GlcNac) was released as glucosamine. Compositional monosaccharide patterns were compared with a standard monosaccharide profile composed of fucose, glucosamine, galactose, glucose and mannose, respectively. Fuc indicates fucose; GlcN, glucosamine; Gal, galactose; Glc, glucose; Man, mannose; GP-2, standard glycopeptide; GA, aglycosylated chimeric anti-CD137 mAb; GG, glycosylated chimeric anti-CD137 mAb; CTM, cetuximab; HuIgG1, polyclonal human IgG1.

Table 1 Compositional monosaccharide quantification of IgG1-Fc

IgG1-Fc	MONOSACCHARIDE MOLAR RATIOS DETERMINED BY HPAEC-PAD ANALYSIS[a]			
	FUC	GlcN	GAL	MAN[b]
Bi-antennary GP[c]	na	3.86	2.08	3.00
HuIgG1	0.95	3.50	0.80	3.00
GG	0.56	3.60	1.40	3.00
GA	na	na	na	na
CTM	1.10	5.20	1.90	3.00

Fuc indicates fucose; GlcN, glucosamine; Gal, galactose; Man, mannose; na, not applicable; HuIgG1, polyclonal human IgG1; GG, glycosylated chimeric anti-CD137 mAb; GA, aglycosylated chimeric anti-CD137 mAb; CTM, cetuximab.

[a]Fc fragments were hydrolyzed in 2M trifluoroacetic acid (TFA) at 100°C for 4 hours. The hydrolysates were lyophilized and subject to Dionex HPAEC-PAD analysis.

[b]For quantification, the peaks corresponding to the different monosaccharides were integrated and peak areas were normalized to molar quantity by comparison with bi-antennary GP. The molar ratios were normalized by setting mannose as 3.

[c]Bi-antennary GP represents a standard glycopeptide isolated from hen's egg yolk that contains a full-size bi-antennary complex type N-glycan with the following molar ratios of monosaccharides: Man:Gal:GlcN = 3:2:4.

Based on these calculations, the molar ratios of the monosaccharide composition of all IgG1-Fc are summarized in Table 1. GG showed much less fucosylated N glycan than CTM and huIgG1. Interestingly, CTM showed a higher content of GlcNAc (Man:GlcN = 3:5) than GG and huIgG1 (Man:GlcN = 3:4), suggesting that this mAb may have an additional bisecting GlcNAc. These observations suggest that the low fucose content on GG might contribute to its enhanced ability to induce CD137 on human NK cells.

CD137 expression on human NK cells is associated with phenotypic markers of activation and the release of pro-inflammatory cytokines

In order to begin to understand the function of CD137 on human NK cells, we sought to associate CD137 expression with cellular activation, as characterized by NK cell surface expression of CD69 and CD54 and pro-inflammatory cytokine secretion. CD137 expressing NK cell cultures were associated with increased levels of CD69 and $CD54^{bright}$ expression with the highest levels of CD69 expression in cultures with huIgG1 and GG mAb (range 32%-62% and 36%-86% respectively; P=0.0361 for GG). $CD54^{bright}$ expression varied from 4%-17% expression in cultures with huIgG1 (P=0.0186) to 10%-24% expression in cultures with GG mAb (P=0.004) as indicated in Figure 5A-B.

Because activated human NK cells are known to secrete pro-inflammatory cytokines, we evaluated the supernatants of NK cell cultures for IFN-γ and TNF-α. Levels of TNF-α and IFN-γ in cultures containing immobilized GG, were significantly increased (P=0.0468 and P=0.0477, respectively) in comparison to cultures with immobilized huIgG1, CTM or soluble mAb (Figure 5C-D). The levels of IFN-γ and TNF-α declined over time; only remaining detectable in wells with immobilized GG after 72 hours (data not shown). In conclusion, these data suggest that Fc dependent CD137 expression is associated with phenotypic markers of activation and pro-inflammatory cytokine release.

Degranulation precedes CD137 expression on human NK cells and results in less efficient lysis of NK sensitive tumor targets by CD137 expressing human NK cells

While Fc-FcγRIII interactions induce NK cell activation resulting in the up-regulation of phenotypic markers of activation and the release of pro- inflammatory cytokines, they do not reveal the capability of these NK cells to lyse targets cells. ^{51}Cr release assays were used to determine the lytic capacity of CD137 expressing and non-CD137 expressing NK cells.

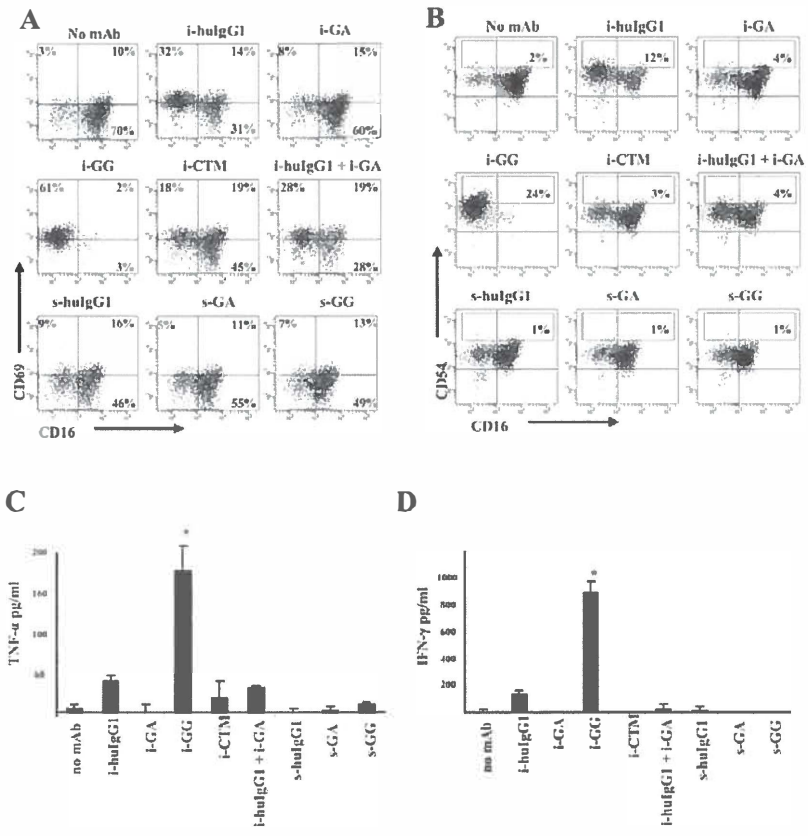

Figure 5. CD137 expression on human NK cells associates with phenotypic markers of activation and pro-inflammatory cytokine secretion. Gates were set around $CD56^{+}$ (NK) cells and numbers in the dot plots indicate the percentage of NK cells expressing indicated surface marker. CD137 expression on IL-2 stimulated NK cells associates with increased levels of CD69 **(A)** and $CD54^{bright}$ **(B)** expression in cultures with immobilized huIgG1 and GG mAb. Pro-inflammatory cytokines were determined by ELISA in supernatants after 24 hours of cultures. **(C)** TNF-α levels were significantly (*P=0.0468) increased in cultures with immobilized GG mAb. **(D)** IFN-γ levels were significantly (*P=0.0477) increased in cultures with immobilized GG mAb. Immobilized mAb culture conditions and soluble mAb culture conditions are indicated with i- and s- respectively. Experiment shown represents one of six individual experiments. Statistical analysis is based on six experiments; CD69 expression levels are significantly increased in NK cell cultures with i-GG (P=0.0361) and $CD54^{bright}$ expression levels are significantly increased in NK cell cultures with i-huIgG1 (P=0.0186) and i-GG (P=0.004) as compared with i-GA. HuIgG1 indicates polyclonal human IgG1; GA, aglycosylated chimeric anti-CD137 mAb; GG, glycosylated chimeric anti-CD137 mAb; CTM, cetuximab.

Surprisingly, the lytic capacity of CD137 expressing NK cells was significantly diminished (P=0.0054) in comparison to non-CD137 expressing NK cells (Figure 6A). Importantly, influence on lytic capacity mediated through CD137-CD137L interactions is unlikely given the fact that K562 tumor targets did not express CD137L as determined by flow cytometry (data not shown). In order to understand

this observation, the temporal relationship between CD137 expression and NK cell degranulation, as measured by CD107a expression (19) was determined. We observed that in the presence of immobilized GG mAb, IL-2 stimulated NK cells express high levels of the cytolytic surface marker CD107a (range 17%-43%; P=0.0109 compared to immobilized GA) after 6 hours of culture (Figure 6B) while CD137 cell surface expression peaks between 12-24 hours of culture. These data suggest that degranulation and CD137 expression on human NK cells are discordant which is reflected in the lytic activity against K562 tumor targets.

Discussion

The critical findings of these studies are that (i) immobilized Fc enhances the expression of CD137 on IL-2 stimulated NK cells, (ii) the levels of CD137 expression are dependent on patterns of Fc glycosylation known to impact Fc interactions with FcγRIII, and (iii) the ability to induce CD137 expression is independent of the antigen specificity of the Fab region. Importantly, our experiments do not specifically address the therapeutic utility of targeting the CD137 pathway on CD137 expressing NK cells, which is perhaps best studied using CD137L. Our findings shed new light onto a secondary function of mAbs against co-stimulatory molecules and demand caution when describing mAbs as being "agonistic" if their function in *in vivo* and *in vitro* studies is assessed with intact Fc regions.

Knowledge of the functional import of CD137 on human NK cells is primarily derived from murine studies. It is now clear that IL-2 stimulated murine NK cells express CD137 *in vivo* and that, in some but not all cases, these cells are required for the antitumor effects of agonistic mAbs against CD137 (2,3,6). The function of CD137/CD137L interactions on murine NK cells is not completely understood, yet it appears that CD137 ligation enhances NK cell proliferation, cytokine secretion and may provide a helper function in the induction of cytotoxic T lymphocytes (CTL); thereby bridging the innate and adaptive immune system in mice (20,21). Correlate studies suggest that *human* NK cells play a pivotal role in the regulation of both innate and adaptive immune responses, yet limited information is available regarding CD137 expression and its role in modulating these interactions (22). For example, it has been demonstrated that low levels of CD137 (~2.02%) can be induced on $NKB1^+$ cells when PBMC are exposed to Doxorubicin, Bleomycin or Mitomycin, although it remains uncertain if the small $NKB1^+CD137^+$ cell population depicted in this study, represents T cells, NK cells, NKT cells or γδT cells (23). Similarly, from a functional perspective, human NK cells transfected

with a construct containing the *intracellular* domain of CD137 directly linked to an anti- CD3/CD19 chimeric receptor are more effective than NK cells transfected with anti-CD3-CD19 chimeric receptor alone, in both killing leukemic cells and producing IFN-γ and GM-CSF (24).

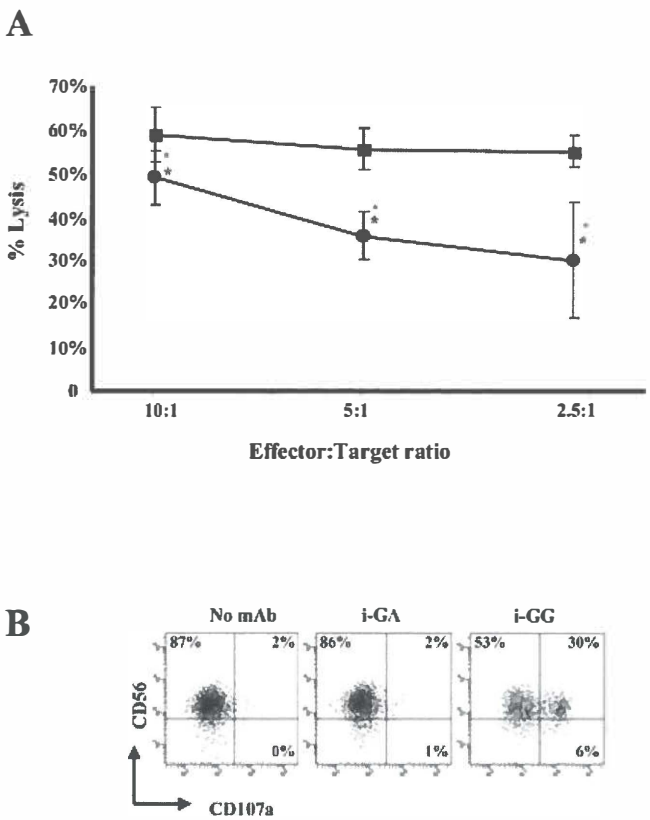

Figure 6. CD137 expressing human NK cells lyse NK sensitive tumor targets less efficient than non-CD137 expressing human NK cells. (A) IL-2 stimulated NK cells were cultured for 24 hours in the presence of immobilized GG mAb (●) or immobilized GA mAb (■) and subsequently used in a 4 hour ^{51}Cr release assay to determine lytic activity against K562 target cells. Non-CD137 expressing NK cells (■) and CD137 expressing NK cells (●) were added at the indicated effector-to-target ratio's (E/T). Data represents mean and standard deviation of two independent experiments. Lytic activity of CD137 expressing NK cells (i-GG cultured) was significantly diminished as compared with non-CD137 expressing NK cells (i-GA cultured) with *P=0.0054. **(B)** Expression of degranulation marker CD107a on IL-2 stimulated NK cells after 6 hours of culture with i-GG. Gates were set around live cells based on forward- and site scatter plots and numbers in the dot plots indicate the percentage of NK cells expressing CD107a. Experiment shown represents one of four individual experiments. Statistical analysis is based on four experiments performed with P=0.0109 for i-GG compared to i-GA. Immobilized mAb culture conditions are indicated with i-. GG indicates glycosylated chimeric anti-CD137 mAb; GA, aglycosylated chimeric anti-CD137 mAb.

Our experiments using various chimeric IgG1 antibodies with different Fc regions, support the novel concept that interactions between Fc and putative FcγRs on the surface of human NK cells directly modulate the CD137 co-stimulatory pathway independent of mAb antigen specificity. Because NK cells express CD16 and CD32 exclusively, it is likely that the observed effects are secondary to ligation of these receptors (25). Importantly, it is unlikely that the ability of GG and GA mAb to block CD137-CD137L interactions influenced our results as CD137L is only reported to be expressed on activated macrophages, dendritic cells and B cells and not on human NK cells (3,26). In addition, similar CD137 expression levels were observed in NK cell cultures in which the CD137 specific Fab fragment was absent (NK cell cultures with GG/Fc). While the up-regulation of CD137 did not appear to be augmented by cross-reactivity of anti-CD137 specific Fab regions, these differences are difficult to ascertain *in vitro*. Our findings offer new insight into the role of antibody Fc fragments in mediating CD137 based NK cell responses.

Consistent with the idea that Fc-FcγR interactions play an important role in regulating CD137 expression on human NK cells, we found that for GG and CTM, the levels of CD137 expression were inversely associated with fucose content within the N-glycan of the Fc region. Furthermore, while CD137 expression levels were enhanced in the presence of 10 fold increases in CTM concentration, CD137 expression levels did not increase in the presence of immobilized GG at concentrations over 10ug/ml. These data are consistent with previous observations that low-fucose IgG1 mAbs enhance NK cell activation at lower mAb density (13). Interestingly, we did not observe an inverse correlation between CD137 expression levels and fucosylated N-glycan in cultures with huIgG1. This observation may reflect the fact that the huIgG1 used in our experiments is polyclonal, resulting in potential enhancement of CD137 expression levels through Fab binding. The higher GlcNAc contents in CTM (Man:GlcN = 3:5) in comparison to GG and huIgG1 (Man:GlcN = 3:4) implicates the presence of a bisecting GlcNAc in the N-glycan of this mAb. It is reported that the addition of bisecting GlcNAc enhances ADCC much less efficiently than the presence or absence of fucose (27). Our observation that, regardless the higher GlcNAc content, CTM is less efficient in inducing CD137, phenotypic markers of activation and pro-inflammatory cytokines is in agreement with these previously published reports. Previous studies have demonstrated that Fc-FcγRIII cross-linking induces NK cell activation resulting in the loss of FcγRIII receptor expression and cytokine release (10) (e.g. IFN-γ and TNF-α). We found a direct association between GG cross-linking and acquisition of an activated NK cell phenotype characterized by CD69 and $CD54^{bright}$

expression. This pattern of activation was inversely associated with FcγRIII expression. However, our studies did not rule out that possible FcγRIII loss could have been artifactual, secondary to the interference of IgG1 with FcγRIII binding.

Interestingly, the number of live NK cells was substantially decreased in cultures with GG. This observation is likely secondary to the specificity of GG for CD137 and two potential mechanisms may be involved. First, GG interaction with CD137 may induce ADCC among CD137 expressing NK cells and/or second, GG interactions may either block or deliver a signal through CD137. These mechanisms are not mutually exclusive, and both could potentially play a role. In addition to displaying phenotypic markers characteristic of NK activation, cultures with high levels of CD137 expression demonstrated the pro-inflammatory cytokines IFN-γ and TNF-α. Furthermore, GG stimulated NK cells were functionally capable of degranulation as evidenced by high levels of CD107a expression (28). As degranulation precedes CD137 expression, reflected by diminished lytic capacity against NK sensitive tumor targets, this may suggest that CD137 expressing human NK cells play a role in immunomodulation rather than exhibiting direct lytic functions. Importantly, because there is a substantial time-interval between the intracellular expression of CD107a and cytokines (maximum at 6 hours) and the surface expression of CD137 (maximum at >12 hours) we cannot ascertain whether CD137 expressing cells are directly responsible for the observed differences.

In summary, our findings that immobilized Fc induces CD137 expression on IL-2 stimulated NK cells are pivotal to the interpretation of studies using anti-CD137 mAbs with an Fc region capable of binding FcγRs. Specifically, under these circumstances, effects previously attributed to CD137 interactions with the Fab region, must now be re-evaluated as potentially related to Fc induced expression of CD137 on NK cells with subsequent effects due to (i) direct interaction of CD137L with Fc induced CD137, (ii) direct stimulation of Fc induced CD137 by agonistic anti-CD137 mAbs or (iii) antibody blockade of interactions between Fc induced CD137 and CD137L.

Acknowledgement

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3

CD137 promotes proliferation and survival of human B cells

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Abstract

CD137 (4-1BB) mediated co-stimulation plays an important role in directing the fate of antigen stimulated T cells and NK cells, yet the role of CD137 in mediating B cell function is currently unknown. Here we report CD137 is expressed both *in vitro* on anti-Ig stimulated peripheral blood B cells and *in vivo* on tonsillar B cells with an activated phenotype. *In vitro*, CD137 expression is enhanced by CD40 stimulation and IFN-γ, and is inhibited by IL-4, IL-10 and IL-21. The expression of CD137 on activated human B cells is functionally relevant, as engagement with its ligand (CD137L) at the time of activation stimulates B cell proliferation, enhances B cell survival, and induces secretion of TNF-α and TNF-β. Our study suggests CD137 co-stimulation may play a role in defining the fate of antigen-stimulated human B cells.

Introduction

CD137 (4-1BB/ILA) is a member of the TNF receptor superfamily and is predominantly found on activated T cells and NK cells (1-3). CD137 ligand (CD137L) is present on APCs including dendritic cells, macrophages, monocytes and B cells (4, 5). Stimulation of CD137, through either its natural ligand or agonistic antibody, induces potent antitumor immunity (6-8), yet also effectively ameliorates disease severity in several mouse models of autoimmunity, including systemic lupus erythematosus (SLE) (9), chronic graft versus host disease (10), collagen induced arthritis (11, 12), inflammatory bowel disease (13) and experimental autoimmune encephalitis (14). Thus, immunotherapeutics targeting CD137 represent promising new approaches to a wide array of distinct immune disorders.

The explanation for the apparent disparity between the ability to promote tumor rejection and treat autoimmune disease appears to be predicated on CD137-mediated manipulation of T cell function. Specifically, in conceptually overlapping experimental models of autoimmunity, CD137 ligation induces T cell deletion or hyporesponsiveness (10, 13), stimulation of $CD4^+CD25^+$ antigen-specific regulatory T cell subsets (15) or proliferation of antigen-specific $CD8^+$ $CD11c^+$ T cells which suppress $CD4^+$ T cell responses (11). This CD137-mediated immune modulation of T cells is postulated to be mechanistically responsible for observed changes in B cell function, including diminished isotype-specific antibody responses and changes in B cell survival. For example, in both murine and primate models, administration of agonistic antibodies against CD137, reduces T cell-dependent antibody production, and CD137 deficient mice demonstrate reduced IgG2a and IgG3 responses to keyhole limpet hemocyanin (KLH) (16-18). Additionally, the importance of CD137-CD137L co-stimulation in B cell survival is evidenced in CD137L transgenic mice, where B cells are noted to decline in absolute number with advancing age (19). Importantly, murine B cells do not express CD137, while human B cells are reported to up-regulate CD137 in response to anti-IgM stimulation (1, 20, 21). Therefore, it is uncertain whether murine-based animal models will accurately predict clinical response to CD137 manipulation. Considering the importance of B cells in antitumor immune regulation and autoimmunity (22, 23), it is striking that the function of CD137 on human B cells has not been elucidated.

The goal of our study was first to evaluate which external signals regulate CD137 expression on human B cells and second to define the biological effect of CD137-

mediated co-stimulation on human B cells. We demonstrate that the expression of CD137 on human B cells *in vitro* is initiated by BCR stimulation while CD40 ligation and cytokines provide second tier regulation. Furthermore, we show that CD137 is naturally expressed on B cells in tonsillar tissue *in vivo*, and that this expression is temporally distinct from CD137L *in vitro*. The presence of CD137 on human B cells is functionally relevant since stimulation with human CD137L transfected cell lines at the time of activation, induces proliferation, protects B cells from activation-induced cell death and promotes secretion of TNF-α and TNF-β. Thus, our findings demonstrate that CD137 co-stimulation may play a role in defining the fate of antigen-stimulated human B cells.

Materials and Methods

Cells

Buffy coats from healthy donors were purchased (Biological Specialty Corporation, Colmar, PA) and PBMC were prepared by density centrifugation (Ficoll-Paque, Amersham). B lymphocytes were purified from PBMC by negative selection using a B cell isolation kit II (Miltenyi Biotec, Auburn, CA) and T lymphocytes were purified by positive selection using CD3 microbeads (Miltenyi Biotec), according to the manufacturer's instructions. Purity of cell separations were typically >98% for B and T lymphocytes with less than 0.2% contamination of $CD3^+$ T cells in purified B cell populations (as assessed by flow cytometry). For the isolation of naïve and memory B cell subsets, $CD19^+$ cells were positively selected using a CD19 multisort kit (Miltenyi Biotec), followed by separation of $CD19^+CD27^+$ (purity >80%) and $CD19^+CD27^-$ (purity >90%) cell subsets using CD27 microbeads (Miltenyi Biotec), according to the manufacturer's instructions.

Tonsils were obtained with informed consent from patients undergoing routine tonsillectomies at the University of Maryland Medical Center. Cells were mechanically homogenized in complete medium, and mashed through a 40-μm sieve to clear the cell solution from tissue fragments and cell clusters. The mononuclear cells were isolated by Ficoll-Paque density centrifugation.

B cell activation experiments

All *in vitro* cell cultures were performed in RPMI 1640 (Cellgro) supplemented with 10% FCS (Atlanta biologicals), 1% penicillin/streptomycin, 1% HEPES and 1% Glutamax (all from Life Technologies).

Stimulation of PBMC: 2×10^6 PBMC were stimulated with 2μg/ml of PWM (Sigma), 5μg/ml of PHA (Calbiochem), 25ng/ml of PMA/1μg/ml of Ionomycin

(Sigma), 2.5μg/ml of CpG (InvivoGen) or 5μg/ml of LPS (Sigma) in 24-well plates. After 72 hours, PBMC were harvested, washed and assessed for CD137 expression by flow cytometry.

B/T cell co-culture experiments: Purified B cells (1×10^6) and T cells (2×10^6) were co-cultured and stimulated with PWM under the conditions described above. Direct B/T cell interactions were inhibited by the addition of transwell membranes. In brief, 2×10^6 T cells were added to the upper chamber of a transwell plate (polyester membranes 6.5-mm, 0.4-μm, Corning Costar) and 1×10^6 B cells were added to the lower chamber.

Anti-CD40L blocking experiments: To block CD40-CD40 ligand (CD40L) interactions, various concentrations (1-20μg/ml) of purified mouse anti-human CD40L (BD Biosciences) mAb or isotype control mAb were added to PBMC and B/T cell co-cultures.

BCR-mediated stimulation experiments: Purified B cells or B cell subsets were cultured at a concentration of 1×10^6/ml in a 48-well or 96-well flat bottom plate. B cells were activated with 10μg/ml anti-Igs F(ab')$_2$ fragments (goat anti-human IgA+IgG+IgM (H+L), Jackson ImmunoResearch Laboratories), with or without the addition of 1μg/ml purified goat anti-human CD40 antibody (R&D Systems). The following human recombinant cytokines were used to evaluate their impact on B cell stimulation: 100U/ml of interleukin-2 (IL-2) (Proleukin, Chiron Corporation, Emeryville, CA), 20ng/ml of IL-4 (R&D Systems), 1ng/ml of IL-6 (BD Bioscience), 50ng/ml of IL-10 (eBioscience), 50ng/ml of IL-15 (R&D Systems), 100ng/ml of IL-21 (Biosource International Inc., Camarillo, CA), 500U/ml of IFN-γ (eBioscience) and 50ng/ml of TNF-α (BD Bioscience).

Real-Time RT-PCR Analysis

Total RNA was isolated using a SV Total RNA Isolation System (Promega) and cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences). Real-time quantitative PCR (Q-PCR) was performed using a 7500-Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with specific primers, probes and software (Applied Biosystems). The level of CD137 mRNA was quantified based on a titrated standard curve co-run in the same experiment and calibrated with the expression level of glyceraldehyde-3-phosphate dehydrogenase. All samples were done in triplicate.

Western blot analysis

Proteins were separated by 4%-20% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham). Membranes were blocked and incubated

overnight at 4°C with goat anti-human CD137 antibody (R&D Systems). Murine mAb against β-actin was used as an internal control. Membranes were then washed and subsequently incubated with horseradish peroxidase–conjugated secondary antibody. The protein bands were visualized by chemiluminescence (GE Healthcare).

Flow Cytometry

Peripheral blood and tonsillar B cells were stained with directly conjugated mouse anti-human mAbs against CD3, CD19, CD20, CD32, CD69, CD86, CD95, CD137, CD137L, IgD, IgM (all BD Bioscience), CD5, CD23, CD25, CD27, CD38 and CD71(all eBioscience). Directly conjugated mouse IgGs were used as isotype controls. Labeled cells were acquired on a LSRII flow cytometer and analyzed with FACS Diva (BD Biosciences) and Winlist (Verity Software House) Software.

To evaluate cell proliferation by flow cytometry, B cells and separated B cell subsets were labeled with CFSE (Alexis Biochemicals), according to the manufacturer's instructions. CFSE labeled cells were cultured as described above and indicated in appropriate figure legends. At day 3 or day 4 of culture, cells were harvested, stained with indicated cell surface markers and analyzed by flow cytometry. The total number of mitoses per a total of 100 gated cells was calculated using the formula: no. of mitoses= $\sum(Xn*100-Xn*100/2n)$, where X is the percentage of cells that underwent n divisions (24).

Cell cycle analysis was performed using a BrdU-FITC flow kit (BD Biosciences). In brief, activated purified B cells were pulsed with 10μM BrdU. After 4 hours, cells were harvested and cell surface stained with mAbs indicated in the figure legends, followed by intracellular staining with anti-BrdU mAb. 7-AAD was used to evaluate DNA content. B cell apoptosis was determined by staining with Annexin V/7-AAD (Annexin V-PE apoptosis detection kit I, BD Biosciences), according to the manufacturer's recommendations. Accucount particles (Spherotech) were added before analyzing samples to obtain accurate absolute cell numbers which were calculated by the manufacturer's instructions.

Immunohistological Analysis.

OCT-embedded sections (5μm) of tonsil were fixed for 15 minutes in 4% paraformaldehyde and washed with PBS for 15 minutes. After incubating with 10% normal human serum, the sections were double-stained for 45 minutes with the following primary antibodies: goat anti-human CD137 (2μg/ml; R&D systems)/mouse anti-human CD20 (1:500; eBioscience) and goat anti-human CD137/FITC-mouse anti-human IgD (1:20; BD Biosciences). Tissue sections were

washed with PBS and blocked with 5% rabbit serum. Each section was then incubated with secondary antibody: Alexa Fluor 594 conjugated rabbit anti-goat IgG, F(ab')2 fragment (1:1000; Invitrogen)/Alexa Fluor 488 conjugated rabbit anti-mouse IgG, F(ab')2 fragment (1:1000; Invitrogen) for 30 minutes. Slides were then washed and counterstained with DAPI (KPL) for 10 minutes. To control for nonspecific binding, control stains with isotype-matched primary antibodies were included. Tissue sections were viewed with a Nikon Eclipse E6000 fluorescent microscopy and were photographed with a Retiga 4000 camera (Q-Imaging).

Functional B cell studies

Generation of CD137L transfectants: Human CD137L cDNA was obtained by RT-PCR from total RNA extracted from human PBMC and sub-cloned into a mammalian expression vector (pcDNA3.1, Invitrogen, Huntsville, AL). P815 cells were transfected with human CD137L using Lipofectamine (Invitrogen). After selection with G418 (800μg/ml) for 1–2 weeks, drug-resistant cells were FACS sorted for CD137L expression. CD137L positive cells were further cloned by limiting dilution. A clone with high levels of CD137L expression (hereafter called P815-CD137L) was selected and used in subsequent functional B cell experiments. P815 cells transfected with vector alone were used as a negative control (hereafter called P815-mock).

CD137-CD137L interaction experiments: gamma-irradiated (100 gy) P815-CD137L cells or P815-mock cells were cultured with purified human B cells for 3-7 days. All cytokines and stimuli were added at the initiation of culture and are indicated in the figure legends.

^{3}H-TdR incorporation assays: Purified B cells were seeded at 2×10^5/well in triplicate wells in a 96 well flat-bottom plate and stimulated as described under "B cell activation experiments" in the presence of irradiated P815-mock or P815-CD137L cells. To block the interaction of CD137 with CD137 ligand, 10μg/ml of soluble CD137 protein (Prospec, Rehovot Isreal) was added at the initiation of culture. ^{3}H-TdR (37 Kbq/well) was added 16 hours before completion of the experiment and thymidine incorporation was measured using a liquid scintillation counter (Wallac).

Induction of apoptosis: Activation-induced cell apoptosis was evaluated by culturing B cells with anti-Ig alone, or in combination with IFN-γ or anti-CD40 as described under "B cell activation experiments".

Cytokine ELISA: Supernatants were collected 24-48 h after cultures were initiated. The concentrations of IL-4, TNF-β (eBioscience), IL-6, IL-10 (BD Bioscience),

IFN-γ and TNF-α (Diaclone) were measured by a standard ELISA following the manufacturer's protocol.

Statistical analysis

A repeated measures model was used to compare CD137 expression under different conditions (reagents) to account for potential intra-donor correlation. The calculation for the repeated measure model was implemented in SAS PROC MIXED. Residual diagnosis such as Q-Q plots were used to check the normality and model goodness of fit. The model reduces to t-test and paired t-test when there was only one sample per donor in each group.

Results

CD137 is expressed on human B cells following activation and expression is regulated by interactions between CD40-CD40L and pro-inflammatory cytokines.

We first characterized activating signals required to induce expression of CD137 on human B cells by stimulating whole PBMC with various mitogenic stimuli. Human B cells were found to up-regulate CD137 in the presence of PWM (12.05±9.76%, n=18, figure 1A) while stimulation with PHA, LPS, PMA/Ionomycin or CpG were not effective (data not shown). Because PWM is recognized to activate both T cells and B cells, we next sought to determine if CD137 expression on human B cells is T cell-dependent. We observed that purified B cells did not up-regulate CD137 in the presence of PWM (data not shown). However, PWM stimulation of cultures containing isolated T cells and B cells, induced B cell associated CD137 expression (19.78±10.52%, n=10). This expression of CD137 is cell-to-cell contact dependent since B cells separated from T cells by a transwell membrane, did not up-regulate CD137 (figure 1B). As the CD40-CD40L co-stimulatory pathway is integral to T cell-dependent B cell function, we defined the relative import of CD40-CD40L interactions in the up-regulation of CD137 on B cells. The addition of escalating concentrations of CD40L mAb to both PWM stimulated PBMC and purified T/B cell co-cultures significantly reduced the percentage of CD137 expression ($P<0.0001$) in a dose-dependent fashion (figure 1C). To confirm the significance of CD40-CD40L interactions in the induction of CD137, purified B cells were stimulated with anti-Ig in the presence or absence of anti-CD40 stimulating antibody. Resting (non-stimulated) B cells and B cells stimulated with anti-CD40 antibody alone did not up-regulate CD137. However, B cells stimulated with anti-Ig up-regulated low levels of CD137, while CD137 expression was dramatically enhanced at both

mRNA and protein levels with concurrent CD40 ligation (figure 1D-F). These data demonstrate that CD137 is induced on human B cells following BCR stimulation and that expression levels are enhanced by interactions between CD40 and CD40L. Importantly, CD137 expression levels on B cells varied among different healthy individuals ranging from 3.2% to 30.7% (n=10; median=13.9%) and from 10.7% to 62.2% (n=14; median=43.1%) upon stimulation with anti-Ig or anti-Ig/anti-CD40, respectively. It is notable that the predominant CD137 band differed between anti-CD3 stimulated T cells and anti-Ig/anti-CD40 stimulated B cells (figure 1F). Specifically, activated B cells display a dominant band at approximately 28 kD, while activated T cells have a dominant band at a size of approximately 40 kD. Importantly, activated B cells also have an identical 40 kD subdominant band. These distinct protein sizes might reflect cell specific post-translational modifications of CD137 or the presence of isoforms in different cell types.

Because cytokines are recognized to influence human B cell function, we evaluated the impact of cytokine stimulation on CD137 expression. Purified B cells were stimulated with anti-Ig/anti-CD40 antibodies in combination with defined cytokines recognized to mediate B cell function. IL-2, TNF-α, IL-6 and IL-15 did not directly affect CD137 expression on human B cells. However, co-culture of anti-Ig/anti-CD40 stimulated B cells with IFN-γ dramatically enhanced ($P<0.0001$) the percentage of CD137 expressing cells while IL-4 ($P<0.0001$), IL-10 ($P=0.0188$) and IL-21 ($P=0.0037$) induced the opposite effect (figure 2A). Similar to the effects of CD40 ligation, the ability of IFN-γ to enhance CD137 expression is dependent upon BCR signaling (figure 2B-C), since B cells stimulated with IFN-γ alone or in combination with anti-CD40 did not up-regulate CD137. These data suggest that exposure to antigen is required for CD137 expression and that CD40 signaling and select cytokines provide a second level of regulatory control for CD137 expression on human B cells.

CD137 is preferentially expressed on activated B cells of naïve origin

As a first step in characterizing the function of CD137 on human B cells, we compared the cell surface phenotype of $CD137^+$ and $CD137^-$ B cells. Anti-Ig/anti-CD40 stimulated $CD137^+$ B cells demonstrated elevated levels of CD71, CD86, and CD95 but diminished expression of CD32 (figure 3A). Interestingly, while $CD137^-$ B cells expressed small amounts of CD27, this marker was virtually absent on $CD137^+$ B cells. Since CD27 distinguishes naïve B cells ($CD19^+CD27^-$) from memory B cells ($CD19^+CD27^+$), we evaluated if CD137 was differentially up-regulated on these distinct populations. Naïve and memory B cells were purified

based on their levels of CD27 expression, cultured in the presence of anti-Ig/anti-CD40, and harvested at defined time intervals. In both cell populations, CD137 expression was present on day one and reached a peak at day three. By day seven, the expression of CD137 returned to baseline. Overall, the percentage of $CD137^+$ B cells was higher in cultures originating from $CD27^-$ versus $CD27^+$ B cells at every time point tested (figure 3B) with P=0.0014 on day four.

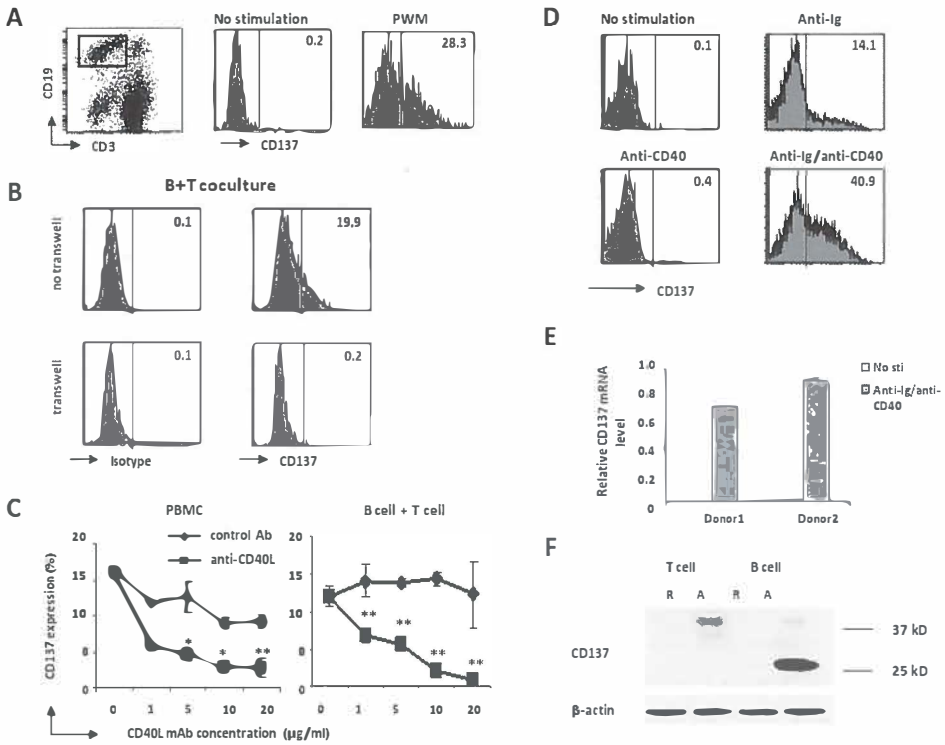

Figure 1. CD137 is induced on human B cells following activation and expression level is enhanced by interaction between CD40 and CD40 ligand. CD137 is up-regulated on B cells following PWM stimulation of PBMC from healthy individuals (n=18). A representative FACS analysis is shown as percentage of CD137 expression on gated B ($CD19^+CD3^-$) cells in whole PBMC **(A)**. B cells require T cell contact in order to up-regulate CD137 during PWM stimulation. Isolated B cells and T cells from healthy individuals (n=10) were co-cultured in the presence of PWM with or without transmembrane for 3 days **(B)**. Histograms indicate percentage CD137 expression on gated B ($CD19^+$ $CD3^-$) cells from one donor. Anti-CD40L mAb blocks CD137 expression on PWM activated B cells in PBMC and B/T cell co-cultures in a dose-dependent fashion **(C)**. Data are presented as mean ± SD and are representative of 5 individual experiments. CD137 is induced on human B cells upon BCR stimulation with enhancement of cell surface expression level following CD40 ligation with agonistic anti-CD40 antibody **(D)**. Histograms indicate percentage of CD137 expression on B cells. The isotype control was used to define the gate in all FACS analyses. Expression of CD137 mRNA **(E)** and protein **(F)** in anti-Ig/anti-CD40 activated B cells from two different donors was assessed by quantitative RT-PCR and Western blot. R indicated resting; A, activated; *P<0.05; **P<0.0001 median =13.9%) and from 10.7% to 62.2% (n=14; median=43.1%) upon stimulation with anti-Ig or anti-Ig/anti-CD40, respectively.

These data demonstrate that following anti-Ig/CD40 ligation, CD137 is preferentially, but not exclusively, found on activated naïve B cells. Because CD137 was not found on non-stimulated peripheral blood B cells from healthy donors, we next sought to determine the expression of CD137 on B cells in secondary lymphoid organs. *Ex vivo* analysis of human tonsillar B cells revealed that 0.8±0.3% (n=6) of total B cells expressed CD137 (figure 4A). Consistent with the *in vitro* phenotype in peripheral blood B cells, these CD137 expressing tonsillar B cells exhibit a phenotype associated with cell activation, e.g. elevated expression of CD23, CD5, CD86, CD71 and CD95. Additionally, $CD137^{+}$ tonsillar B cells are surface IgM^{hi}, $CD38^{+/-}$ and surface $IgD^{+/-}$ (Figure 4B). The majority of $CD137^{+}$ B cells are found in the germinal center and fewer cells are found in the follicular mantle zone (figure 4C-D).

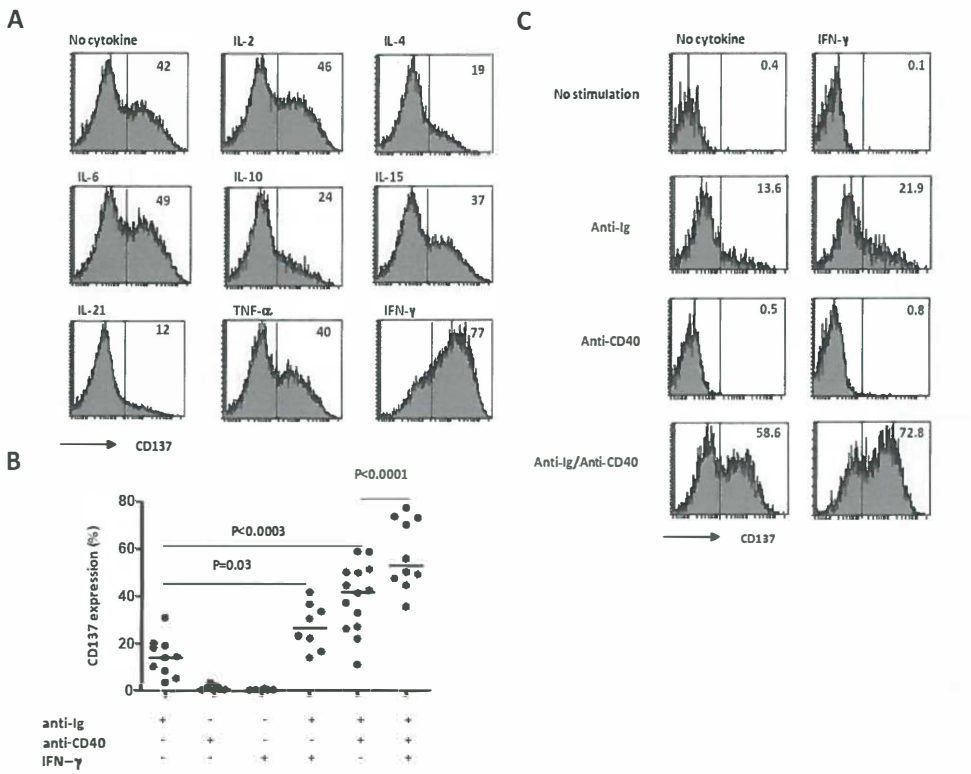

Figure 2. Cytokines provide a second level of regulatory control for the expression of B cell associated CD137. Purified B ($CD19^{+}$) cells were anti-Ig/anti-CD40 stimulated in the presence of no cytokine, IL-2, IL-4, IL-6, IL-10, IL-15, IL-21, TNF-α or IFN-γ. After 3 days of culture, B cells were harvested and assessed for CD137 expression by flow cytometry **(A)**. CD40 ligation and/or IFN-γ enhance CD137 expression levels on human B cells but primary B cell receptor stimulation is required **(B)**. A representative FACS analyses from one donor is shown as percentage of CD137 expression on B cells **(C)**. Data are representative of at least 10 individual experiments. The isotype control was used to define the gate in all FACS analyses.

The differentiation of human B cells into effector immunoglobulin secreting cells requires cell division (25, 26). Therefore, we sought to determine if CD137 expression on human B cells is associated with B cell division. Based on CFSE dilution, we observed that higher levels of CD137 are expressed on divided (figure 5A) compared to non-divided B cells (figure 5B). Specifically, CD137 is predominantly expressed on divided memory B cells. In contrast, both divided and non-divided naïve B cells express high levels of CD137 although higher CD137 expression levels are found on naïve B cells with low CFSE intensity. Importantly, the expression of CD137 does not solely rely on B cell division since CD137 is present on the B cell surface prior to cell division and blocking of B cell division with mitomycin C does not abrogate the ability of anti-Ig/anti-CD40 activated B cells to up-regulate CD137 (data not shown). Furthermore, cell cycle analysis confirmed that more $CD137^+$ B cells (30%) are present in the S phase than $CD137^-$ B cells (15%; figure 5C).

CD137 ligation enhances B cell proliferation

To expand upon the observation that CD137 expression on B cells is associated with B cell activation and cell division, we evaluated whether CD137 co-stimulation of activated B cells induces proliferation. In order to study the effect of CD137 co-stimulation on human B cells, P815 cell clones expressing human CD137L were generated. The P815-CD137L cells were recognized by both mouse anti-human CD137L mAb and human CD137 fusion protein, confirming cell surface expression of CD137L and the ability to interact with the human CD137 receptor, respectively (data not shown). Purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ or anti-Ig/anti-CD40 in the presence of P815-CD137L or P815-mock cells. In comparison to B cell cultures with P815-mock, the presence of P815-CD137L significantly enhanced ^{3}H-TdR incorporation in all three culture conditions (figure 6A). As both IFN-γ and anti-CD40 activate B cells, the baseline of ^{3}H-TdR incorporation without P815 transfectants were significantly higher in the group with anti-Ig/anti-CD40, followed with anti-Ig/IFN-γ, in comparison with anti-Ig only stimulation. The specificity of the observed effects was confirmed in CD137-CD137L blocking experiments, where the addition of soluble human CD137 protein to anti-Ig stimulated B cell cultures completely abrogated the observed differences (figure 6B). These data suggest that the observed changes in thymidine incorporation are specifically mediated through CD137-CD137L interactions. Subsequent studies using CFSE dilution analysis confirmed that CD137 enhanced anti-Ig activated B cell proliferation in all five donors tested ($P=0.0076$) (figure 6C). Specifically, anti-Ig activated B cells only completed one

cell division in the presence of P815-mock whereas in the presence of CD137 co-stimulation (P815-CD137L), cells completed two or more divisions. Additionally, CD137L stimulated B cells exhibited a greater percentage of cells in the S phase of the cell cycle compared to those cultured with P815-mock (figure 6D). When B cells were activated with anti-Ig/anti-CD40, a significantly higher percent of cells (66.1%) underwent cell division compared with anti-Ig stimulation (23.9%).

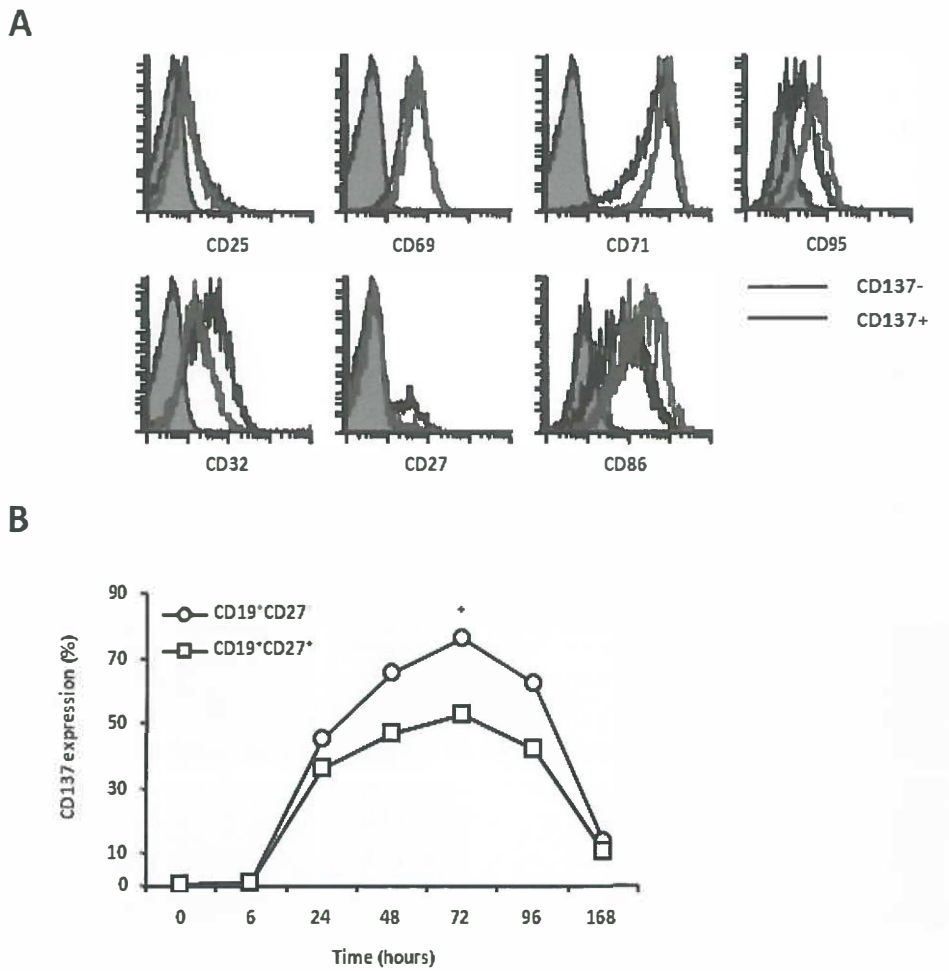

Figure 3. CD137 is preferentially expressed on activated B cells of naïve origin. Purified human B cells were activated with anti-Ig/anti-CD40. After 3 days, B cells were harvested and CD137 expressing B cells and non-CD137 expressing B cells were assessed for cell surface phenotype by flow cytometry **(A)**. Histograms show surface expression of indicated markers on $CD137^+$ B cells and $CD137^-$ B cells. Filled peaks represent isotype controls. Data are representative of 5 individual experiments. Purified human B cells were separated into naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells and subsequently stimulated with anti-Ig/anti-CD40. CD137 surface expression was determined at indicated time-points **(B)**. Data shown are representative of 5 individual experiments. *$P<0.05$.

However, in this setting, CD137 stimulation does not further enhance the cell division and cell cycle progression (data not shown). These data suggest that the initial B cell activation signal determines the net effect of CD137-mediated B cell proliferation.

CD137 ligation promotes human B cell survival

Based on our observation that CD137 ligation in B cell cultures exposed to anti-Ig/anti-CD40 enhanced thymidine incorporation, yet failed to induce clear cell division and cell cycle progression, we postulated that CD137 may provide a survival advantage for B cells. In order to test this hypothesis, purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ, or anti-Ig/anti- CD40 in the presence of mock or CD137L transfected P815 cells.

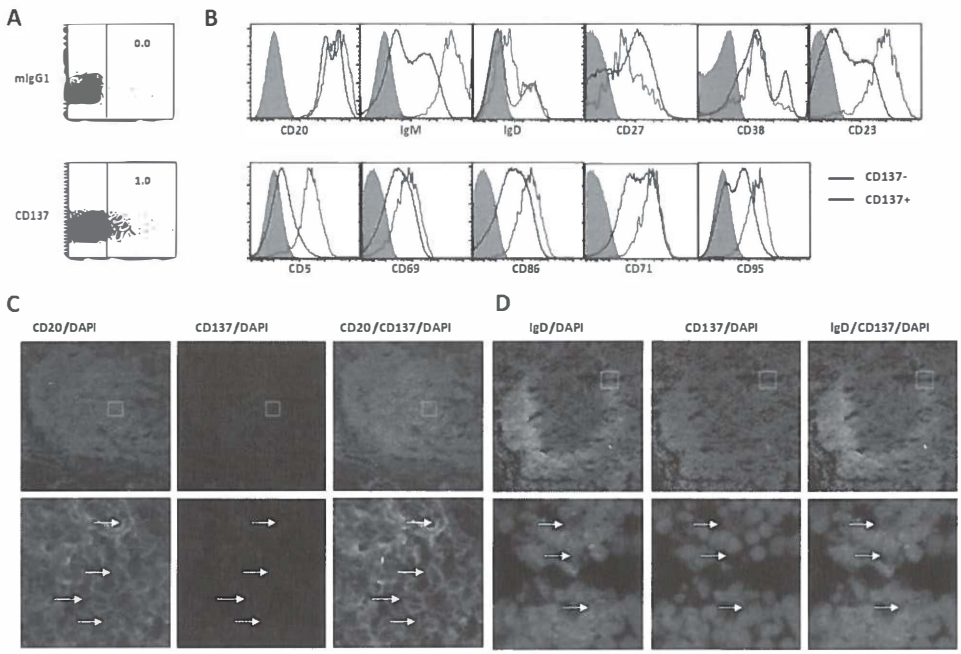

Figure 4. *Ex vivo* expression of CD137 in tonsillar B cells. Tonsillar mononuclear cells were analyzed for CD137 expression on B cells by FACS. B cells ($CD19^+CD3^-$) were gated and the percentage of CD137 expression on B cells is indicated in a dot plot **(A)**. Data are representative of 6 individual experiments from different donors. The phenotype of $CD137^+$ B cells was analyzed in comparison to $CD137^-$ B cells **(B)**. Filled peaks indicate isotype controls. Data are representative of 5 individual experiments. Three-color immunohistochemistry of human tonsil sections was used to identify the localization of CD137 expressing B cells. The upper panel shows a tonsillar germinal center (original magnification ×20; CD20, green; CD137, red; DAPI, blue) **(C)** and follicular mantle zone (IgD, green; CD137, red; DAPI, blue) B cells **(D)**. The indicated area from germinal centers and follicular mantle zones were enlarged to identify the $CD20^+/CD137^+$ and $IgD^+/CD137^+$ cells (lower panel).

Cell survival was assessed at various time points by flow cytometric staining with Annexin-V and 7-AAD. In the presence of P815-CD137L, both the percentage and absolute number of survived B cells (as determined by Annexin-V$^-$/7-AAD$^-$) improved in all three culture conditions (figure 7A-B) at day four and day six.

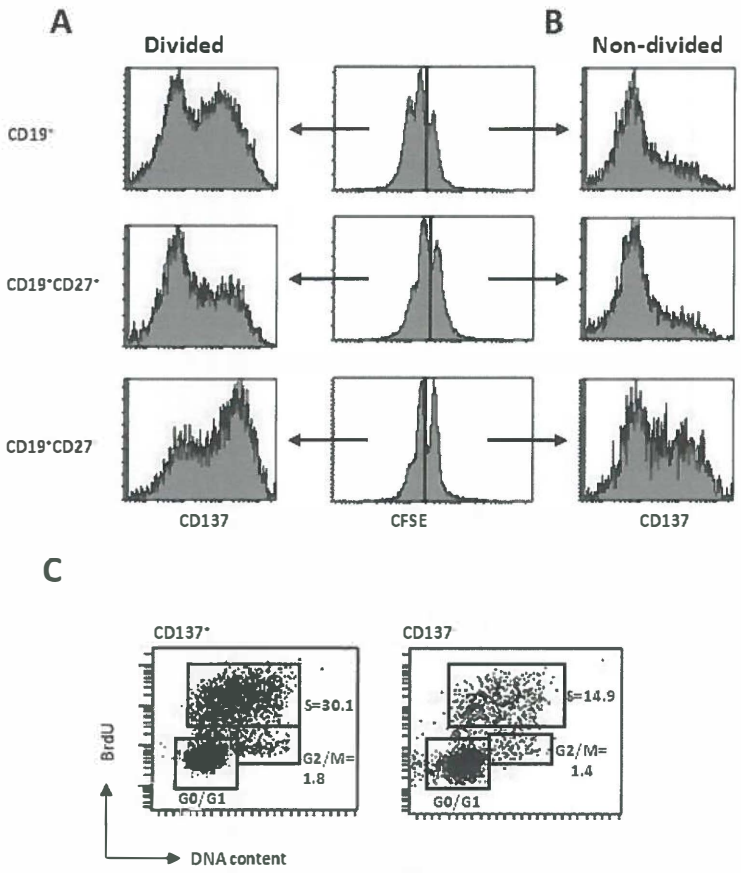

Figure 5. CD137 expression is associated with enhanced B cell proliferation. High levels of CD137 are expressed on divided human B cells. Non-separated (bulk; CD19$^+$), memory (CD19$^+$CD27$^+$) and naïve (CD19$^+$CD27$^-$) B cells were CFSE labeled and stimulated with anti-Ig/anti-CD40. After 4 days, cells were harvested and analyzed for CFSE dilution and CD137 expression by flow cytometry. Based on CFSE dilution, divided and non-divided B cell subsets were gated and analyzed for CD137 surface expression. Histograms indicate percentage of CD137 expression on indicated subset of divided **(A)** and non-divided **(B)** B cells. Data are representative of at least 5 individual experiments. CD137 expression is associated with cell cycle progression in anti-Ig/anti-CD40 stimulated human B cells. Anti-Ig/anti-CD40 stimulated B cells were cultured for 3 days and pulsed with BrdU for 4 hours. Cell cycle status of CD137 expressing (CD19$^+$CD137$^+$) and non-CD137 expressing (CD19$^+$CD137$^-$) B cells was determined by flow cytometry using anti-BrdU and 7-AAD **(C)**. Data are representative of 3 individual experiments.

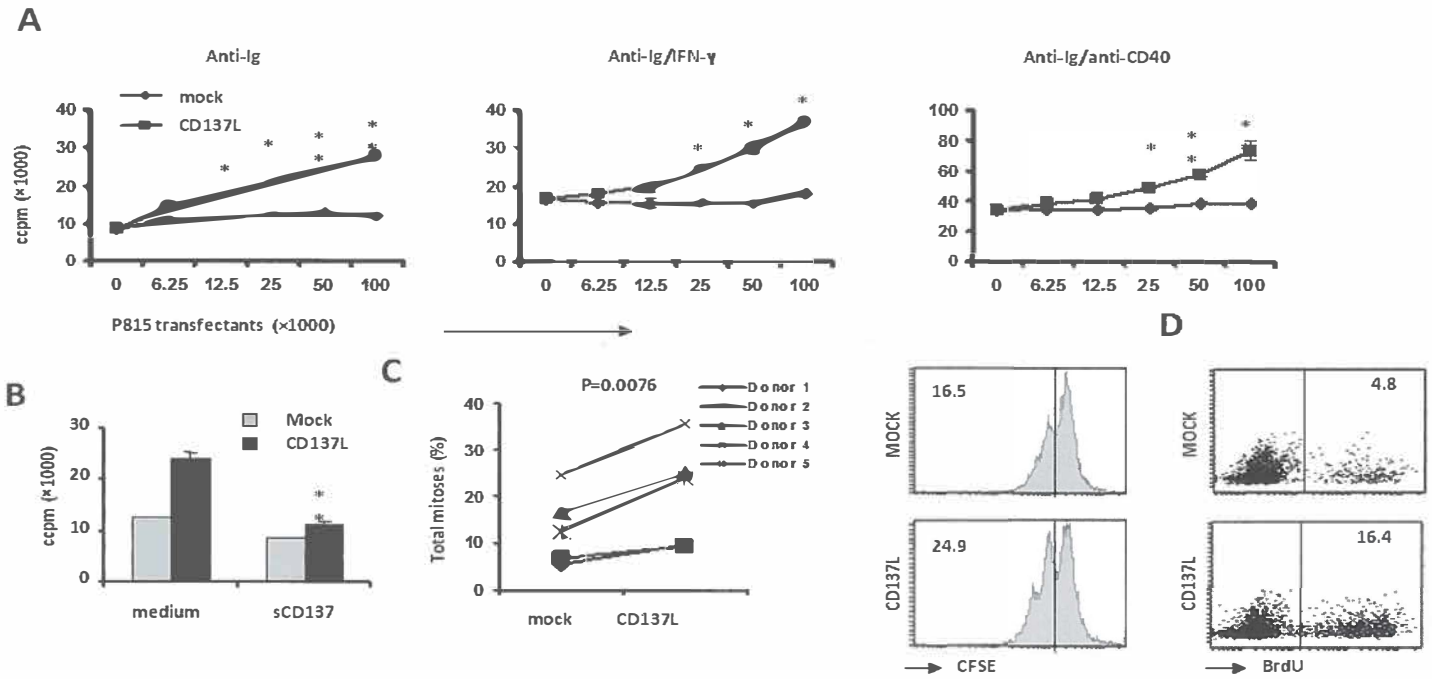

Figure 6. CD137 ligation stimulates B cell proliferation. Purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ or anti-Ig/anti-CD40 in the presence of various numbers of irradiated P815-mock or P815-CD137L as indicated in the figure. Thymidine incorporation was measured after 3 days (anti-Ig, anti-Ig/IFN-γ) or 4 days (anti-Ig/anti-CD40) of culture **(A)**. Data are presented as mean ± SD of triplicate wells and are representative of 5 experiments. Without activation, the baseline thymidine incorporation (CCPM) of B cells ranges from 100 to 200. The addition of soluble CD137 (sCD137) protein to cultures with anti-Ig activated B cells abrogates the enhanced thymidine incorporation by P815-CD137L activated B cells. Bar graph shows thymidine incorporation in anti-Ig stimulated B cell cultures with P815-mock or P815-CD137L after the addition of sCD137 **(B)**. Data are presented as mean ± SD of triplicate wells and are representative of 4 individual experiments. Anti-Ig stimulated B cells exhibit enhanced cell proliferation in the presence of P815-CD137L cells at day 4 as measured by CFSE dilution analysis. A representative FACS analysis of a CFSE dilution assay (donor 3) is shown **(C)**. Values indicate the percent of mitoses of gated B lymphocytes. Anti-Ig stimulated B cells have more cells in the S phase of the cell cycle in the presence of irradiated P815-CD137L cells in comparison to P815-mock controls **(D)**. Data are representative of 4 individual experiments. *P<0.05, **P<0.001

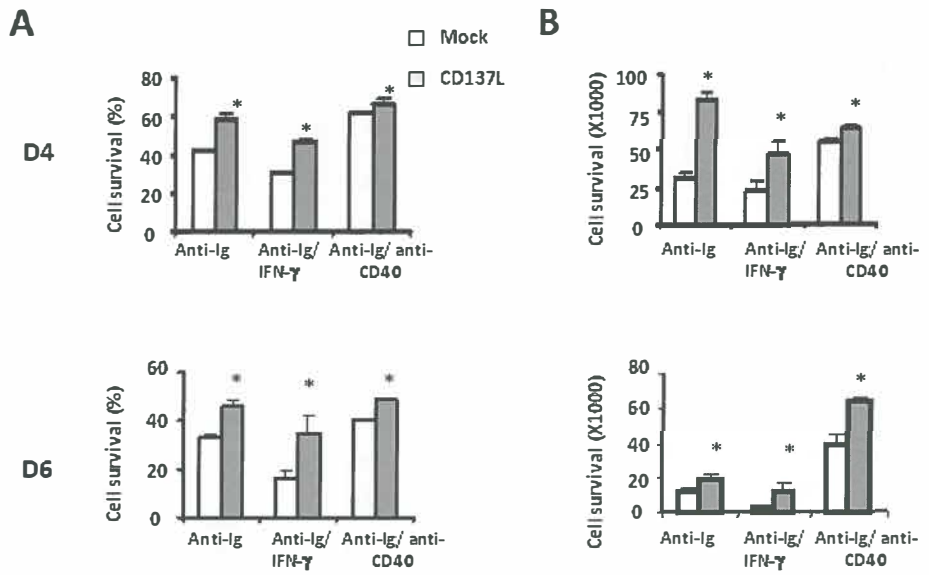

Figure 7. CD137 ligation enhances B cell survival. Purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ and anti-Ig/anti-CD40 in the presence of P815-mock or P815-CD137L cells. The percentage **(A)** and absolute number **(B)** of B cell survival was assessed by flow cytometry at culture day 4 and day 6. B cells were identified from P815 cells according to FSC *vs.* SSC as well as CD19 staining. Annexin-V and 7-AAD staining was used to distinguish the survived cells (Annexin-V$^-$/7-AAD$^-$) from apoptotic and dead cells. Data are shown as mean ± SD of pooled data from 5 donors for anti-Ig and anti-Ig/IFN-γ stimulation and 6 donors for anti-Ig/anti-CD40 stimulation. Bar graphs represent B cells survival in cultures with P815-mock or P815-CD137L cells. *P<0.05

However, despite the significant higher CD137 expression level induced by anti-Ig/anti-CD40 stimulation, the difference in the CD137 mediated enhancement of survival in anti-Ig/anti-CD40 activated B cells appears less than the B cells activated by anti-Ig alone, or anti-Ig/IFN-γ. In fact, anti-Ig/anti-CD40 activated B cells exhibited a higher survival rate in comparison with anti-Ig alone, or anti-Ig/IFN-γ in the absence of CD137 ligation, which is consistent with previous observations (27, 28), that CD40 provides a survival signal for B cells.

CD137 ligation induces secretion of TNF-α and β

It is known that B cells produce effector cytokines upon activation (29, 30). In order to further define the function of CD137 stimulation on activated B cells, we stimulated B cells with anti-Ig/anti-CD40, in the presence of irradiated CD137L or mock transfected P815 cells and measured cytokine release by ELISA. No significant differences in the production of IL-4, IL-6, IL-10 or IFN-γ were detected between mock and CD137L stimulated B cells.

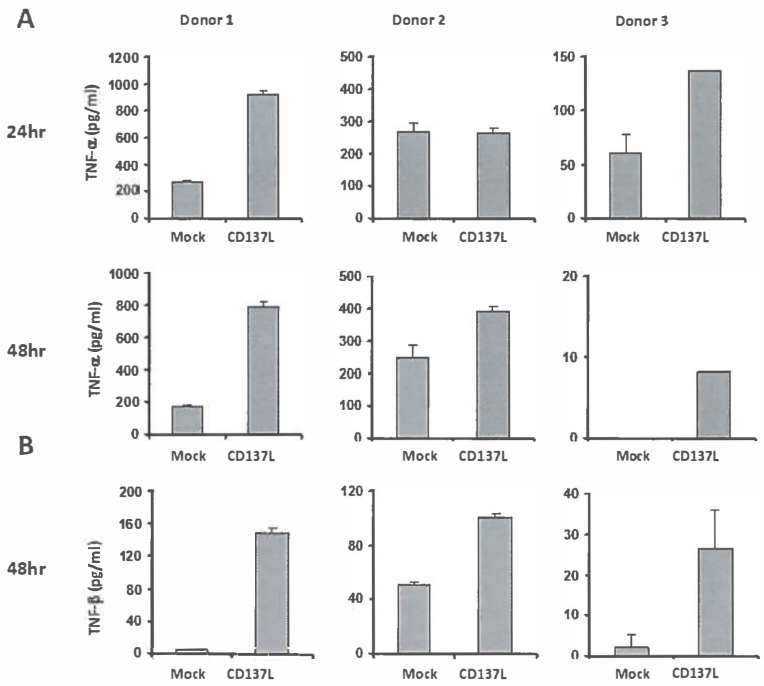

Figure 8. CD137 ligation enhances TNF production by anti-Ig/anti-CD40 activated B cells. Purified B cells from 3 donors were activated with anti-Ig/anti-CD40 in the presence of irradiated CD137L or P815-mock cells. Cell supernatants were collected at 24 and 48 hours and cytokine levels were detected by ELISA.

However, B cells stimulated with CD137L demonstrated profound increases in both TNF-α and TNF-β secretion in each of the 3 donors tested (figure 8). These data are particularly interesting given the recognized role of TNF antagonists in the treatment of autoimmune diseases, such as rheumatoid arthritis and SLE, in which activated B cells are postulated to play an important role in disease pathogenesis.

Discussion

Here we demonstrate that despite the reported absence of CD137 on murine B cells, CD137 is expressed on human B cells. Our studies indicate that CD137 is expressed on activated B cells following BCR stimulation. Cognate help from T cells through CD40-CD40L interaction and/or cytokines are important for regulation of CD137 expression on human B cells. Among the cytokines tested, only the Th1 cytokine IFN-γ enhanced CD137 expression, while IL-4, IL-10 and IL-21 inhibited CD137 expression. Importantly, neither anti-CD40 stimulation nor cytokine alone were capable of inducing B cell expression of CD137 in the absence of BCR stimulation. In addition, polyclonal stimulation of human B cells with

CpG, which stimulates TLR9-mediated B cell proliferation and differentiation in the absence of antigen, fails to up-regulate CD137 (data not shown). These data suggest that (i) CD137 expression on human B cell is tightly controlled, (ii) strictly dependent upon antigen encounter and that (iii) the BCR serves as the initial "switch" which enables up-regulation of CD137 on the B cell surface.

Similar to T cell-associated CD137, B cells transiently up-regulate CD137 with detectable cell surface levels after 24 hours of activation, maximal expression levels by day three and a return to baseline levels by day seven. This study demonstrates that $CD137^+$ B cells are phenotypically associated with enhanced expression of CD5, CD23, CD71, CD86 and CD95 while CD32 expression is decreased, which implies that $CD137^+$ B cells are highly activated. Furthermore, divided B cells express higher levels of CD137 compared to non-divided B cells, although CD137 expression is not dependent on B cell division. Interestingly, upon anti-Ig/anti-CD40 stimulation, naïve B cells are more prone to enhanced CD137 expression than memory B cells. We postulate that CD137 co-stimulation may be especially important for naïve B cell regulation, since naïve and memory B cells require different signals for cellular activation and differentiation (31, 32).

The functional effects of CD137-mediated co-stimulation on T cell proliferation and survival are well documented (33-35). Here we demonstrate that CD137 on human B cells mediates analogous functional changes. ^{3}H-thymidine incorporation by anti-Ig, anti-Ig/IFN-γ or anti-Ig/anti-CD40 stimulated B cells is significantly enhanced upon ligation with CD137. Interestingly, B cell division and cell cycle progression are differently affected depending on the initial B cell activation signal. For example, B cell division is enhanced through CD137 ligation on B cells which have been stimulated with anti-Ig alone. In contrast, B cell division and cell cycle are not affected by CD137 ligation among B cells which are stimulated with both anti-Ig and anti-CD40, despite significantly higher CD137 expression levels. Since CD40 signaling is known to mediate B cell proliferation and survival (28), delicate CD137-CD137L mediated enhancements in cell division and cell cycle progression may be masked by the potent proliferative effects of the CD40 signal itself. Despite the fact that various B cell stimuli, e.g. anti-Ig, anti-Ig/IFN-γ and anti-Ig/anti-CD40, impact differently on CD137-mediated B cell proliferation, B cell survival was improved by CD137 ligation in all culture conditions. Specifically, enhanced improvement in B cell survival was observed among B cells activated by anti-Ig alone or in combination with IFN-γ, compared to those activated by anti-Ig/anti-CD40. Overall, our data indicate that CD137-mediated changes in B cell function

depend on initial B cell activation and are the net effect of both CD137-mediated B cell proliferation and B cell survival.

Ex vivo histological analysis of human tonsil showed that the $CD137^+$ B cells mainly localize to the germinal center. Taken together with the observation that CD137 ligation promotes B cell proliferation and survival *in vitro*, we postulate that the interaction of CD137 with CD137L expressed on APC plays an important role during early B cell activation and expansion in the germinal center reaction.
Because B cells are reported to express CD137L (36), we examined the relationship between CD137L and CD137 expression *in vitro* and *ex vivo* (supplemental figure 1). CD137L is expressed during the early stage of B cell activation followed by the upregulation of CD137. In our *in vitro* culture conditions, the majority of cells did not co-express CD137 and CD137L. However, activated B cells at different development stages circulate *in vivo*. We were able to detect both $CD137^+$ and $CD137L^+$ B cells *ex vivo* from human tonsil, though the receptor and ligand are primarily expressed on distinct populations (supplemental figure 2). In this scenario, the interaction of $CD137L^+$ B cells with $CD137^+$ B cells might bi-directionally co-stimulate B cells and mediate cell functional changes, since CD137L can also reverse signals in certain cell types (37-39).

In addition to antibody production and antigen presentation, activated B cells produce cytokines and thus, regulate immune responses (29, 30). Moreover, B cells play a key role in the pathogenesis of diseases such as rheumatoid arthritis demonstrated by the clinical efficacy of targeted B cell depletion (40). In response to CD137 stimulation, the production of TNF-α and TNF-□ is greatly enhanced by anti-Ig/anti-CD40 activated B cells. Both cytokines independently or complementarily enhance immune responses and augment inflammation by targeting various immune cells. Specifically, (i) TNF-α has been established as a central player in the pathogenesis of rheumatoid arthritis (41), (ii) TNF-β is required for the formation of germinal center like structures within the inflamed synovium (42) and (iii) our unpublished studies suggest that the CD137 expression on peripheral B cells is elevated in autoimmunity (e.g. rheumatoid arthritis). Therefore, our data suggest that CD137 mediated B cell stimulation might be involved in the pathogenesis of rheumatoid arthritis. Further investigation on the function of B cell-associated CD137 in autoimmunity is underway.

To the best of our knowledge, the present study is the first to demonstrate that CD137 promotes human B cell proliferation, B cell survival and cytokine production. The CD137 receptor is distinct from other TNF receptor family members that regulate B cell proliferation and differentiation, such as CD40 and BAFF-Receptor (BAFF-R) (43-45). CD40 and BAFF-R are constitutively expressed on B cells whereas the upregulation of CD137 is strictly dependent on the presence of anti-BCR stimulation. Similar to BAFF-R, CD137 co-stimulates the proliferation of B cells in the presence of anti-BCR. In contrast, CD40 stimulates B cell proliferation and differentiation in the absence of anti-Ig. Therefore, it is likely that CD40, CD137 and BAFF-R fulfill distinct functions *in vivo*. However, comparison of the signaling cascade initiated through these receptors requires further investigation.

Finally, our findings have important implications for the clinical translation of CD137 based immunotherapeutic strategies. The disparity of CD137 expression and function between human and murine B cells challenges the use of murine-based disease models for the evaluation of CD137-mediated immune regulation. As a result, targeting the CD137 pathway with therapeutic intent may have unanticipated consequences on human B cell function. In addition, the fact that the expression of B cell-associated CD137 is regulated by many of the factors involved in the pathogenesis of rheumatoid arthritis and SLE (e.g. enhanced CD40 expression and altered cytokine production) suggests that B cell-associated CD137 might be of functional import in these diseases (46, 47).

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4

Epitope mapping of a chimeric CD137 mAb: A necessary step for assessing the biologic relevance of non-human primate models

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Abstract

Antibody-based manipulation of the CD137 (4-1BB) co-signaling pathway is an attractive option for the treatment of cancer and autoimmune disease. We developed a chimeric anti-human CD137 monoclonal antibody (GG) and characterized its function. As a component of planned preclinical studies, we evaluated the binding of GG to activated PBMCs from cynomolgus macaque and baboon against human. Interestingly, GG only recognized human CD137, while a commercial anti-CD137 mAb (4B4-1), recognized activated PBMCs from both human and non-human primates. Subsequent analysis revealed that the amino acid sequence of CD137 is largely conserved between primate species (~95% identical), with the extracellular domain differing by only 9-10 amino acids. Based on these data, we generated mutant constructs in the extracellular domain, replacing nonhuman primate with human CD137 sequences, and identified 3 amino acids critical for GG binding. These residues are likely part of a conformational epitope, as a peptide spanning this region is unable to block mAb binding. These data demonstrate that subtle sequence variations of defined co-stimulatory molecules amongst primate species can be employed as a strategy for mapping residues necessary for antibody binding to conformational epitopes.

Introduction

Recent events such as the TGN1412 trial in 2006, where healthy volunteers became critically ill following the administration of a super-agonistic anti-CD28 mAb, underscore the importance of appropriate animal model choices in preclinical testing of therapeutic agents which alter the function of co-signaling pathways (1-4). The ability of co-signaling molecules to modulate cell function has stimulated significant interest in manipulating these pathways with therapeutic intent. In order to maximize therapeutic benefits while reducing unanticipated side effects, both identification of co-signaling pathways with improved toxicity profiles and development of preclinical tests which more accurately predict potential toxic side effects in humans are mandated (3).

Manipulation of the CD137 (4-1BB) co-signaling pathway can be used to provide a favorable toxicity profile, as shown in previous studies (5). While CD137 is induced on both activated human T cells and NK cells, the ligand, CD137L, is expressed on monocytes, dendritic cells and B cells (6,7). Binding of CD137 to its ligand can result in T cell proliferation and increased cell survival (9). Other studies demonstrated antitumor immunity in mouse models in an NK-dependent fashion (5,10). Unexpectedly, activation of CD137 also ameliorates autoimmune diseases in several murine models including, inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus (11-13). While the mechanism for CD137 antitumor immunity and reduction of autoimmune responses appear distinct, manipulation of this pathway is attractive for the treatment of disease.

In collaboration with GTC Biotherapeutics Inc, we developed a chimeric mouse anti-human CD137 mAb with a human IgG1 Fc, GG. Prior to clinical testing of this antibody, we planned to evaluate its safety in non-human primate (NHP) models. Interestingly, GG effectively bound to human CD137, but failed to recognize either activated cynomolgus macaque (*Macaca fascicularis*) or olive baboon (*Papio anubis*) PBMC. In contrast, another anti-CD137 mAb (4B4-1), bound CD137 from both human and NHPs activated PBMCs. To map the binding site of GG, we first characterized the DNA sequences of CD137 from NHPs including cynomolgus macaques, olive baboons, and rhesus macaques (*Macaca mulatta*). The full-length CD137 from these NHP species demonstrated high homology, differing from human by only 9 amino acids (AA, cynomolgus) and 10 AA (baboon) in the extracellular domain. Based on these data, we generated constructs of the external CD137 domain, replacing individual NHP CD137

sequences with the human equivalent, and identified three amino acids necessary for GG binding. These amino acids are likely to contribute to a conformational rather than a linear epitope, as we were unable to inhibit binding with a synthetic 20 amino acid long peptide that contained the human sequence for the region identified. These data demonstrate that sequence variations between human and NHPs can serve as an effective tool for mapping the binding sites of antibodies against co-stimulatory molecules and provide further insight into the sites of CD137-CD137L interactions.

Materials and Methods

All experimental work related to NHP material was approved by the University of Maryland's Institutional Animal Care and Use Committee (IACUC).

Antibodies

The methods of chimerization and purification of the GG monoclonal antibody was previously published (7). For flow cytometry, GG was directly conjugated with allophycocyanin (APC) by Invitrogen (Molecular Probes Invitrogen, Eugene, OR) and 4B4-1 mAb was purchased from BD (BD Biosciences, San Jose, CA).

Cell culture

Wild type and transfected Chinese hamster ovary (CHO) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% of penicillin/streptomycin (Invitrogen, Carlsbad, CA), Glutamax (Invitrogen, Carlsbad, CA), and HEPES buffer (Mediatech Inc., Herndon, VA). The cells were grown at 37°C in 5% CO_2 atmosphere.

PHA activation

Human, rhesus macaque and baboon PBMC were plated at $2x10^6$ cells/well in 24 well plates, activated with 20μg/ml of PHA and incubated at 37°C, 5% CO_2. After 48 hours, cells were harvested, washed and subsequently stained with CD137 specific mAbs as indicated. In addition, non PHA activated cells were stained for FACS and used as a baseline control.

cDNA preparation

RNA from human PBMC (Biological Specialty Corporation, Colmar, PA) was extracted using a Qiagen RNA kit (Qiagen, Valencia, CA) as per manufacturer's instructions. cDNA of all species was purified from the processed RNAs using

Bio-Rad cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's recommendations.

Primers

The primers for human and NHP used in our study are shown in Table 1. Human specific CD137 primers were designed from the human CD137 sequence (NCBI accession number NM_001561.4). Cynomolgus macaque (*Macaca fascicularis*) and olive baboon (*Papio anubis*) specific CD137 primers were designed based on the rhesus macaque (*Macaca mulatta)* CD137 sequence (NCBI accession number XM_001096166).

PCR amplification

cDNA encoding the extracellular CD137 domain of human and NHP were amplified using polymerase chain reaction (PCR). Briefly, the PCR reaction was optimized using 2μL of cDNA, 0.5mM dNTPs, 1 unit GoTaq polymerase (Promega, WI) and corresponding 1X buffer containing 1.5 mM Mg^{2+}. The PCR conditions include an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 50°C for 45 seconds, and a final extension at 72°C for 10 minutes. The PCR amplified products were run on a 2% agarose gel and visualized with ethidium bromide staining. Where extractions were required, PCR products were obtained using a Qiagen Gel extraction kit (Valencia, CA) according to manufacturer's recommendations.

Subcloning of inserts

All amplified CD137 DNA were cloned into a TOPO TA Cloning® vector (pCR®2.1-TOPO®) (Invitrogen, Carlsbad, CA) according to manufacturer's suggestions. Each insert was confirmed by sequencing at the Biopolymer/Genomics Core Facility, University of Maryland, Baltimore, USA. After characterization the inserts were subcloned into expression vector pcDNA 3.1 using either a PCR based sewing by overlap extension procedure (14) or using existing restriction sites resulting in full-length CD137 constructs.

Transfection

All plasmids used were transfected into a CHO cell line using FuGENE® 6 Transfection reagent (Roche, Indianapolis, IN) according to manufacturer's recommendation. For transient transfection, CHO cells were seeded in a 6 well plate the day before transfection at a density of ~1.5 x 10^5 cells per well in 2 mL DMEM complete media.

Table 1 Primer sequences used for PCR amplification of human and NHP CD137

Name	Sequence[a]	Orientation	PCR specificity
SES 49	5'-CAGCCCTATTGACTTCCA-3'	Reverse	Human full-length 4-1BB
SES 57	5'-ACCTGTGCCAGATTTCAT-3'	Forward	Human full-length 4-1BB
SES73	5'-GCGAAGCTTGCCATGGG-AAACAGCTGTTACAACATAG-3'	Forward	Cyno[b] full-length 4-1BB
SES74	5'-TGCTCAGATTAATGGTGAT-GGTGATGATGCAGTTCACATCC TCCTTCTTC-3'	Reverse	Cyno full-length 4-1BB
SES 84	5'-GGAGGACAGGGACTGCAA-ATTTGATTCCTGTTATTATCACA GAATGTACC-3'	Reverse	Mutate cyno K-50*, I-66*, S-67*, Y-69*and E-75* to R, T, P, F, G, respectively
SES 85	5'-GGTACATTCTGTGATAAT-AACAGGAATCAAATTTGCAGTC CCTGTCCTCC-3'	Forward	Mutate cyno K-50*, I-66*, S-67*, Y-69*and E-75* to R, T, P, F, G, respectively
SES 120	5'-AGTTGTACTGCACTCCAGGGTT TCA-3'	Forward	Mutate cyno I-66*, S-67* and Y-69* to T, P and F, respectively
SES 121	5'-TGAAACCCTGGAGTGCAGTCAC ACT-3'	Reverse	Mutate cyno I-66*, S-67* and Y-69* to T, P and F, respectively
SES 178	5'-ATTTCAGGGTATCACTGCCTGG GGGCAGGA-3'	Forward	Mutate human TI-66*, P67* and F-69* to I, S and Y, respectively
SES 179	5'-ATACCCTGAAATGCAGTCACAC TCTGCATTGCT-3'	Reverse	Mutate human TI-66*, P67* and F-69* to I, S and Y, respectively

[a]Human specific CD137 primers were designed based on NCBI accession number NM_001561.4. Cynomolgus macaque and baboon specific CD137 primers were designed based on the rhesus macaque (Macaca mulatta) CD137 sequence (NCBI accession number XM_001096166.1).
[b]Cyno, cynomolgus macaque
*Numbering begins at the first amino acid after the signal peptide sequence of the human 4-1BB sequence (8).

One μg of green fluorescent protein DNA was used as a control to determine transfection efficiency. After 48 hours of transfection, cells were either harvested for transient analysis or selected for resistance to G-418 at 1mg/mL for 1 week. To obtain clones from the stable transfection, limiting dilution cloning of the transfected cells were maintained in the standard growth medium containing 0.8mg/mL G-418. Stable clones were selected and screened for optimal expression of CD137 using flow cytometry with commercial anti-CD137 mAb (clone 4B4-1).

Flow cytometry

Expression of CD137 was determined by direct staining with mouse anti-human CD137 (4B4-1) or chimeric GG mAb. Directly conjugated human IgG1 and mouse IgG2a were used as isotype controls for the mAbs 4B4-1 and GG, respectively.

Cells were incubated with the antibodies at 4°C for 30 minutes and washed twice with FACS buffer and acquired using a BD™ LSR II flow cytometer and analyzed with BD™ FACS DIVA Software or WinList™ (Verity Software House, Topsham, ME).

Peptide inhibition

A peptide was synthesized identical to the human CD137 protein (Thr^{58}-Ser^{59}-Asn^{60}-Ala^{61}-Glu^{62}-Cys^{63}-Asp^{64}-Cys^{65}-Thr^{66}-Pro^{67}-Gly^{68}-Phe^{69}-His^{70}-Cys^{71}-Leu^{72}-Gly^{73}-Ala^{74}-Gly^{75}-Cys^{76}-Ser^{77}), and was used to block the recognition of the GG mAb to its natural antigen. The peptide, with a calculated molecular weight of 1972.2 g/mol, was titrated from 156.25 µg/mL to 5000 µg/mL (0.08 mM – 2.54 mM). This concentration range is sufficient to saturate the GG mAb at 3.5 µg/mL (0.08 mM). Briefly, various concentrations of peptide were added to 3.5 µg/mL of GG mAb and incubated at 4°C for 30 minutes. CHO transfected human CD137 cells were added to the peptide-mAb mixture and incubated for another 30 minutes at 4°C. Cells were washed once and analyzed by flow cytometry.

Results

GG antibody does not recognize CD137 on activated baboon or rhesus PBMC

To understand the value of NHP models for evaluating the toxicity of our anti-CD137 mAb, we first sought to determine if the antibody bound to activated NHP T-cells. We and others have previously observed that activated PBMC express CD137 (15). FACS analysis of activated PBMC stained with a commercially available anti-CD137 mAb, 4B4-1, or the chimerized anti-human CD137 mAb, GG, revealed that all mAbs equally bound to CD137 on activated human PBMC. However, CD137 expression was only detected on activated PBMC from NHPs by 4B4-1 (Figure 1). Importantly, NHP cells were recognized by 4B4-1 after activation, demonstrating that activation of these cells was effective. These results suggest that sequence differences between human and NHP CD137 molecules could be employed to map the epitope recognized by GG.

Characterization of human, baboon, and rhesus macaque CD137

As a first step towards epitope mapping, we cloned and sequenced CD137 full-length cDNA from both human and NHP. To clone the NHPs, we engineered primers based on either the human (NM_001561.4), or rhesus macaque (XM_001096166) sequences. The forward primers were designed to identify sequences in the region of the putative signal peptide and reverse primers in the predicted C terminus of the protein. RNA from human, cynomolgus macaque and

baboon PBMC were purified, reverse transcribed, amplified using PCR, and the products were cloned and characterized. Amino acid sequences of our human, rhesus and cynomolgus macaque CD137 clones were identical to the available NCBI published sequences (16,17). The novel baboon (*Papio Anubis)* sequence was deposited in GenBank with accession number FJ348360 (BankIt accession number 1139275). At the amino acid level, the full-length CD137 sequence of cynomolgus macaque and baboon differed from human by 4.7% and 5.4%, respectively (Figure 2).

Expression of human, cynomolgus macaque and baboon CD137 constructs in CHO cells

In order to minimize the variability observed in mAb binding in activated PBMC, we established stable cell lines expressing either human or NHP full-length CD137 proteins. This was achieved by transfecting the full-length human, cynomolgus macaque and baboon CD137 cDNA into CHO cells. The stably transfected cell lines were stained with 4B4-1 and GG. Figure 3 shows FACS analysis of CHO transfected human, cynomolgus macaque and baboon CD137 expressing cells.

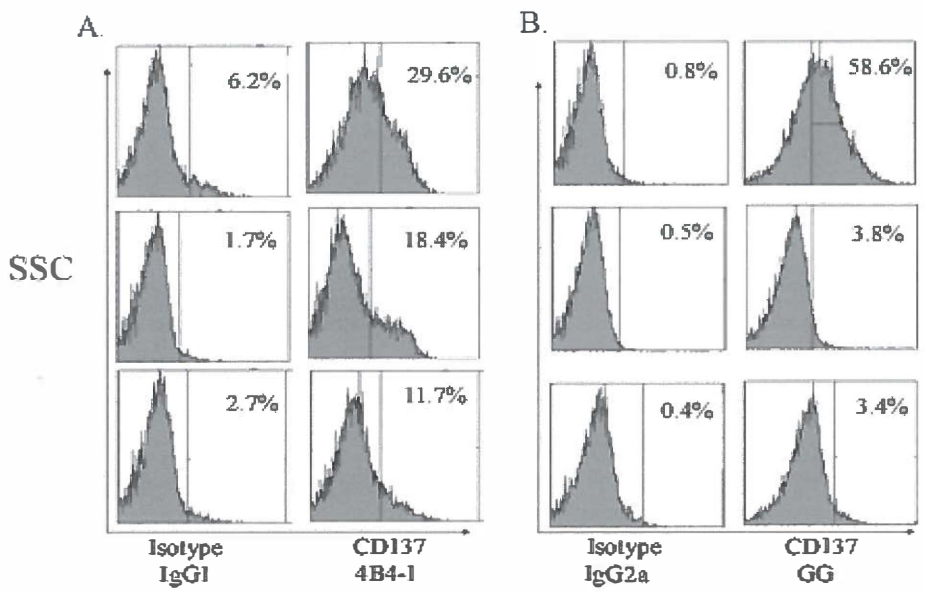

Figure 1. Expression of CD137 on activated PBM from human and NHP. PBMC were activated with PMA/Ionomycin for 48 hours and stained with either isotype control antibodies (A) or with anti-human CD137 monoclonal antibodies (B). The vertical axis is represented by side scatter (SSC). The numbers represent the percentage of $CD137^+$ cells. Gates were set according to the isotype controls as indicated.

Clones demonstrating a high level of anti-CD137 expression using mAb 4B4-1 were selected and tested against GG. Although all transfected clones consistently expressed high levels of anti-CD137 when stained with 4B4-1, only cells transfected with the human CD137 were recognized by GG. Using the full length human and cynomolgus macaque constructs, we designed an approach to identify the epitope uniquely recognized by GG by engineering chimeric constructs (Figure 4).

Glycosylation is not responsible for observed binding differences for GG

Glycosylation of proteins can affect ligand interaction or alter antibody recognition. In the sequence characterization of human CD137, there were two sites (Asn^{103} and Asn^{115}) in the extracellular portion of the protein that scored highly for possible glycosylation (18).

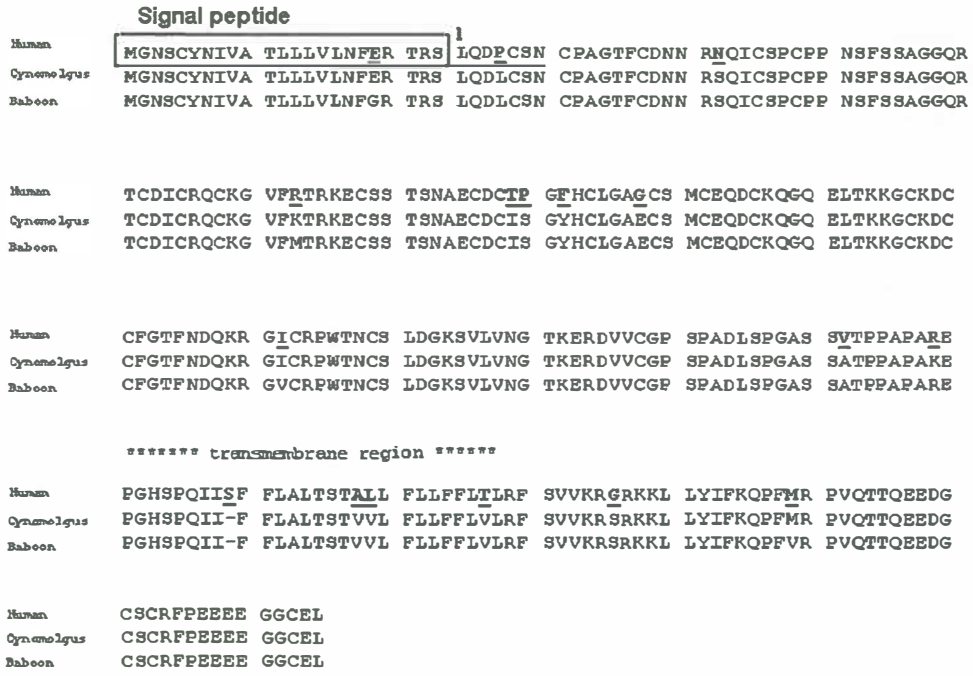

Figure 2. Comparison of human and NHP CD137 sequences. Sequence comparison of full-length CD137 between human and NHP indicate the protein is conserved throughout old world primates and apes. The human sequence is based on Pub Med NM_001561.4 submission. The predicted signal sequence in human is identified in the box and the numbering of the human sequence is based on the mature protein (after removal of the predicted signal sequence (8)). The putative transmembrane region is noted and bounded by asterisk. Differences between human and NHP sequences are identified in bold and underlined.

However, these residues were shared between the human and non-human primate species used in this study. Another site that had a significantly lower probability for glycosylation, Asn^{19}, was only present in the human sequence and differed in NHP (Figure 2). Based on these data, we mutated Ser^{19} in the cynomolgus macaque to Asn^{19}, which is present in the human sequence. FACS analysis demonstrated that the cynomolgus macaque mutated construct was not detected by GG, suggesting that Asn^{19} and glycosylation are not important for anti-CD137 mAb binding (Figure 4, Construct 1).

Binding of GG depends on the amino acid sequence between Arg^{37} and Ile^{109}

To further localize the epitope, we took advantage of the sequence similarity between human and NHP and created a chimeric construct using conserved restriction sites present in both human and cynomolgus macaque at the same position within the sequence. Restriction digestion of both human and cynomolgus macaque CD137 constructs with restriction enzymes PpuMI and SalI permitted the creation of a new construct in which amino acids from Arg^{37} to Ile^{109} of cynomolgus macaque were replaced with those of the human equivalent (Figure 4, Construct 2). Stable clones of transfected construct 2 were stained with the anti-CD137 mAbs and significant binding of chimeric GG to this partially "humanized" cynomolgus construct was observed. These data localize the possible residues responsible for GG binding to the central part of the extracellular region of CD137.

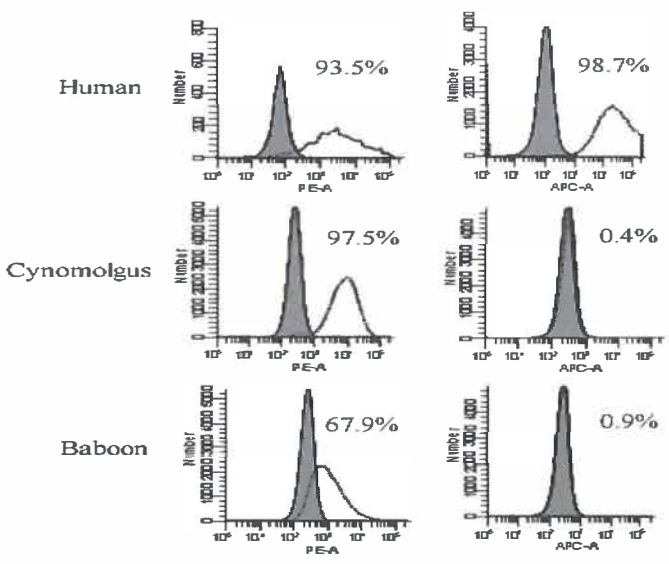

Figure 3. CD137 expression of stably transfected CHO cells with human and NHP CD137 constructs. Stably transfected CHO cells expressing the human and NHP full-length CD137 constructs were stained with either 4B4-1 or GG mAb. The highest CD137 clones (determined by staining with 4B4-1 mAb) were used for studies. Filled histograms represent isotype control mAb; open histograms represent CD137 mAb staining.

Binding of GG antibody is dependent upon amino acids Thr[66], Pro[67] and Phe[69]

To further define the possible binding site(s) of GG, we mutated the three clustered amino acids at position Ile[66], Ser[67] and Tyr[69] of the human equivalent (Thr[66], Pro[67] and Phe[69]) in the cynomolgus construct using gene sewing. This resulting chimeric CD137 cynomolgus construct is recognized by both 4B4-1 and GG (Figure 4, Construct 3). To insure that these residues are responsible for binding, we made the reciprocal construct using human CD137 as the backbone and replaced the three amino acids from cynomolgus macaque with the human equivalent using the same approach. As predicted, the full length human CD137 construct with these three amino acid changes from cynomolgus macaque was not recognized by GG after transfecting into CHO cells (Figure 4, Construct 4).

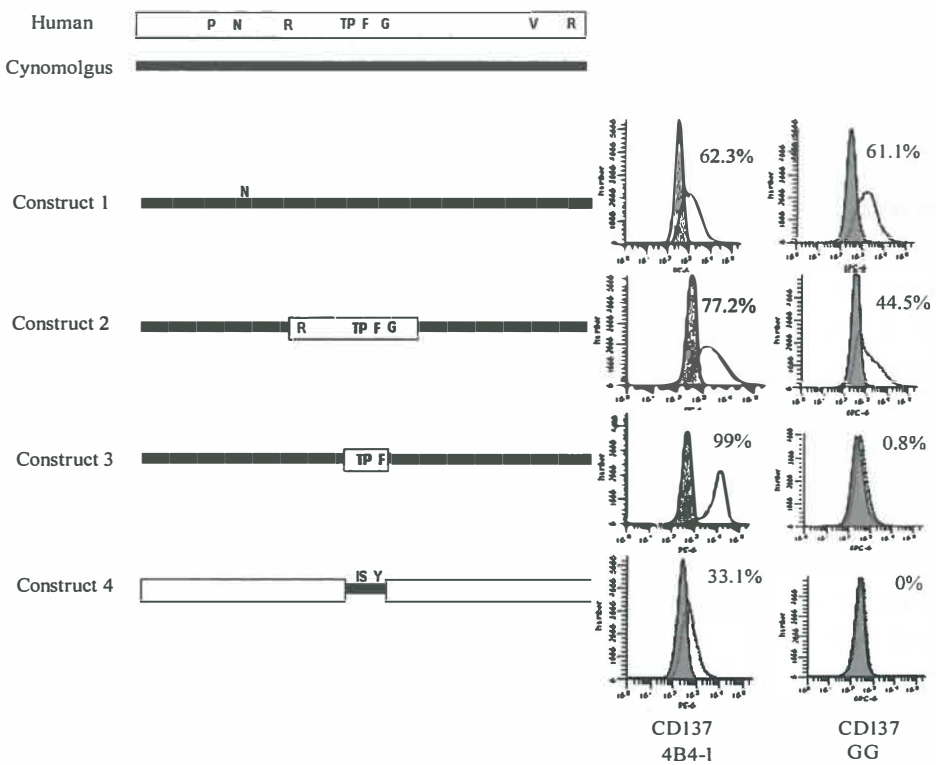

Figure 4. Constructs of the extracellular region of CD137 used in GG mAb epitope mapping. "Chimeric constructs (1-4) were engineered based on the amino acid (AA) sequence dissimilarities found between human and cynomolgus macaque using gene sewing PCR or sequence replacement by restriction enzyme digestions due to conserved restriction sites present in both species. Constructs 1, 2 and 3 made from the cynomolgus backbone, were replaced with equivalent regions of the human CD137. Construct 4 was engineered to contain cynomolgus CD137 AA on a human CD137 backbone as a control to determine if indeed, the indicated 3 AA residues contribute to GG binding. Filled histograms represent isotype control mAb; open histograms represent CD137 mAb staining.

Epitope recognition by GG is not inhibited by a 20 AA peptide spanning residues 58-77

To determine whether the structure of the binding site was a linear epitope, and whether a peptide could be employed for *in vitro* blocking of the antigen, we synthesized a 20 amino acid long peptide identical to the human sequence. This peptide contains the three unique amino acids (Thr^{66}, Pro^{67} and Phe^{69}) that contributed to mAb GG recognition to the CD137 protein. When increasing concentrations of peptide were added to a constant amount of GG mAb, we observed no decrease in antibody binding (Figure 5). In addition, we used both Western blotting with CD137 fusion protein and immunoprecipitation from a CD137 transfected CHO cell lysate with biotinylated GG to detect a non-reduced CD137 (data now shown). However, we were not able to observe the same antibody recognition under reduced conditions. Additionally, incubation with the synthetic peptide had no effect in either reduced or non-reduced conditions, suggesting that GG may only recognize conformational characteristics that are not present in the soluble peptide or after alteration of protein structure.

Discussion

Safe and effective translation of antibodies designed to manipulate co-signaling pathways requires in depth preclinical assessment, which often includes studies in NHP. The relevance of such studies is largely dependent upon the ability of the target reagent to elicit functionally similar responses amongst primate species. In order to understand the potential utility of NHP studies for predicting the safety and toxicity of a novel chimeric anti-CD137 mAb, GG, we performed binding studies on activated T cells from humans, baboons and cynomolgus macaques. While the commercially available anti-CD137 (4B4-1) antibody bound all species comparably, GG bound only to human. In order to understand the differences in binding affinity between these mAbs, we sequenced the cDNA of CD137 from several NHP species and revealed that the CD137 proteins are ~ 95% identical when compared to human. Sequential replacement of cynomolgus macaque with human sequences identified at least three amino acids critical for GG binding. These residues are likely involved in a conformational rather than a linear epitope, as an overlapping peptide was unable to inhibit GG recognition, even at excess molar concentrations. To determine the relevance of planned preclinical NHP studies to evaluate the safety and toxicity profile of GG, we performed binding studies on activated PBMC from rhesus, cynomolgus macaque and baboons. Our initial binding data were difficult to interpret. Specifically, all

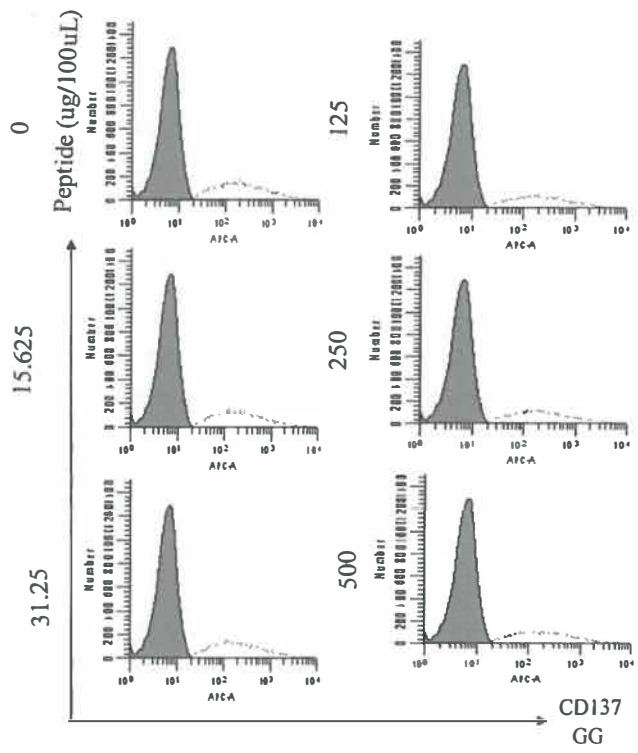

Figure 5. Human peptide sequence does not inhibit binding of GG to CHO-transfected human CD137. A 20 amino acid peptide consisting of the sequence between position 64-83 was used to determine inhibition of GG to CHO-transfected human CD137 cells. CD137 GG mAb was pre-treated with various concentrations of the peptide and then incubated with the human CD137 transfected CHO cells. Filled histograms represent isotype control mAb, open histograms represent peptide/mAb staining.

antibodies clearly bound activated human PBMC, and mAb 4B4-1 also recognized activated PBMC from NHP. However, GG binding to PBMC from NHP was equivocal. To clearly determine the ability of GG to recognize NHP CD137, we generated stable constructs expressing these molecules using the cynomolgus macaque species. While 4B4-1 recognized all NHP CD137 tested, GG failed to bind cells expressing these constructs. These data are particularly relevant to preclinical NHP studies using antibodies against inducible co-signaling molecules as they clearly demonstrate that binding studies must be interpreted with caution, particularly in the absence of true positive and negative controls.

To date, only a limited amount of research has been carried out to investigate the cross-reactivity of CD137 mAbs with NHP species. A recent study using a novel humanized CD137 mAb (H4B4) not only demonstrated cross-reactivity to activated baboon $CD3^+$ and $CD4^+$ T cells, but also indicated that the immune unresponsiveness using the H4B4 mAb was antigen specific, and not by immune suppression (19). Another study using cynomolgus macaques as study model suggests a possible increase in cellular response based on subdominant epitopes of the mAb (20). These data, along with our amino acid sequences of both baboon and

cynomolgus macaque demonstrate that CD137 is largely conserved throughout old world primates and apes. Furthermore, this sequence conservation and the presence of a mAb which recognized CD137 from human but not NHP as well as another that recognized both provided an ideal opportunity for both epitope mapping and as a means to gain insight into potential residues involved in CD137-CD137L interactions.

Our data indicate that residues 66, 67, and 69 in the extracellular domain of the human CD137 are critical for GG binding. The necessity of these residues for antibody recognition was confirmed by mutating the NHP constructs at specific amino acid positions to human, which restored binding affinity. Similarly, analogous mutations in the human construct to cynomolgus macaque eliminated binding. Importantly, because the residues used for producing the mutants were from the human CD137 sequence, it is unlikely that the failure of antibody recognition was secondary to significant structural changes in the protein. Furthermore, the fact that the mutant constructs retained their ability to be recognized by the mAb 4B4-1, suggests that the residues present in human CD137 directly contribute to GG recognition. Using the NCBI database, we found that the only other animal that contains the three amino acids Thr^{66}, Pro^{67} and Phe^{69} responsible for GG recognition is the chimpanzee (accession number XM_001157779). Unfortunately, research using chimpanzees has been severely restricted. Additionally, inherent polymorphisms for CD137 within individuals of a species need to be considered before initiating further studies.

In conclusion, we demonstrate an approach that can be applied to strategically define mAb epitope binding residues necessary for recognition by manipulation of NHP sequences. These findings are particularly relevant to preclinical NHP studies for two reasons. First, the fact that our initial binding studies from activated PBMC were equivocal, demonstrates that activation of native cells may be difficult to interpret. This was overcome in our studies by making stable transfections. Second, in some instances, the high degree of homology between NHP and human co-stimulatory molecules can facilitate rapid identification of both linear and conformational epitopes potentially important for antibody binding. A recent published sequence for cynomolgus CD137 agrees with our AA sequence in this study, with the exception of K156 in our sequence compared to R156 in that study (16). Optimistically, use of this comparative homology strategy for epitope identification, will ensure the relevance of NHP models for preclinical testing of mAbs, and enhance the safety of mAb based clinical trials.

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PART B

NK cell-based immunotherapy and its therapeutic potential in patients with solid malignancies

5

Ex vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity

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Abstract

The possibility that autologous NK cells could serve as an effective treatment modality for solid tumors has long been considered. However, implementation is hampered by (i) the small number of NK cells in peripheral blood, (ii) the difficulties associated with large-scale production of GMP compliant cytolytic NK cells, (iii) the need to activate the NK cells in order to induce NK cell mediated killing and (iv) the constraints imposed by autologous inhibitory receptor-ligand interactions. To address these issues, we determined (i) if large numbers of NK cells could be expanded from PBMC and GMP compliant cell fractions derived by elutriation, (ii) their ability to kill allogeneic and autologous tumor targets by direct cytotoxicity and by antibody-mediated cellular cytotoxicity and (iii) defined NK cell specific receptor-ligand interactions that mediate tumor target cell killing. Human NK cells were expanded during 14 days. Expansion efficiency, NK receptor repertoire before and after expansion, expression of NK specific ligands, cytolytic activity against allogeneic and autologous tumor targets, with and without the addition of chimeric EGFR monoclonal antibody, were investigated.

Cell expansion shifted the NK cell receptor repertoire towards activation and resulted in cytotoxicity against various allogeneic tumor cell lines and autologous gastric cancer cells, while sparing normal PBMC. Blocking studies confirmed that autologous cytotoxicity is established through multiple activating receptor-ligand interactions. Importantly, expanded NK cells also mediated ADCC in an autologous and allogeneic setting by antibodies that are currently being used to treat patients with select solid tumors.

These data demonstrate that large numbers of cytolytic NK cells can be generated from PBMC and lymphocyte-enriched fractions obtained by GMP compliant counter current elutriation from PBMC, establishing the preclinical evidence necessary to support clinical trials utilizing autologous expanded NK cells, both directly and in combination with monoclonal antibodies in future cell-based immunotherapy in select solid tumors.

Introduction

Natural killer cells (NK) were identified more than 30 years ago as a population of lymphokine activated killer cells that showed the ability to kill tumor cells in vitro in the absence of prior immune sensitization of the host (1,4). Over the ensuing years, much has been learned about regulation of their biologic activity and, in particular, their potential use as an immunotherapeutic modality in cancer (5). It has become clear that the biologic activity of NK cells is controlled by a complex repertoire of surface receptors which, upon engagement by ligands on a target cell, signal either an inhibitory or activating response (6). The major inhibitory and activating receptors are products of germ line genes encoding killer cell immunoglobulin-like receptors (KIRs) and in an autologous environment, inhibition of NK cell cytotoxic activity is dominant and governed by epitopes on self HLA class I alleles. In general, cytotoxic activity of NK cells is triggered when the target cell lacks expression of some or all HLA class I molecules; the basis for the "missing self" hypothesis (7). Recognizing the possibility that NK cells have the ability to kill tumors that lack expression of the inhibitory HLA class I alleles, investigators have reported significant antitumor responses in clinical settings of allogeneic stem cell transplantation. Importantly, clinical effects are demonstrated when inhibitory effects are bypassed by utilizing haplo-identical NK cells and best results are achieved when, in addition, KIR-ligand mismatched NK cells are selected (8,9). In turn, this approach requires extensive donor screening and careful depletion of allogeneic T cells from the NK cell product before administration to the host in order to avoid the risk of graft-versus-host disease (GvHD) (10).

The possibility that infusion of autologous NK cells could serve as an effective treatment modality for solid tumors has long been considered (11). However, implementation is hampered by (i) the small number of NK cells in peripheral blood that could be isolated relative to the number of cells that would be required to be effective and the difficulties associated with large-scale production of cytolytic NK cells in compliance with Good Manufacturing Practices (GMP), (ii) the need to activate the NK cells in order to induce NK cell mediated killing of a resident tumor and (iii) the constraints imposed by autologous inhibitory receptor-ligand interactions. The first issue has been addressed in a number of reports that demonstrate that large numbers of NK cells could be expanded from $CD56^+$ cells isolated from peripheral blood mononuclear cells (PBMC) obtained from healthy individuals and patients with hematological malignancies and solid tumors. Expansion was achieved by short term culture with cytokines alone, by cytokines and co-culture with irradiated feeder cells consisting of EBV transformed

lymphoblastoid cell lines or cytokines and co-culture with K562 cells that had been transfected with and express cell membrane-bound IL-15 and 4-1BBL (12-16). In most instances, these expanded cells were generated from NK cells ($CD56^+CD3^-$) isolated from peripheral blood using magnetic beads. The expanded NK cells were highly cytotoxic when tested against variety of target cells that consisted primarily of allogeneic cancer cell lines established from hematologic malignancies (12,17). In addition, a GMP compliant and closed system has successfully been established for the enrichment of monocytes from PBMC using counter current elutriation (18). Besides a highly enriched population of monocytes, lymphocyte-enriched fractions are also obtained. Currently, clinical studies are ongoing utilizing elutriation derived monocytes for large-scale generation of dendritic cells in order to treat a variety of metastatic cancers.

The objectives of this study were to evaluate if the aforementioned strategies could be combined in order to expand large numbers of NK cells from PBMC from normal individuals and patients with various solid tumors. Furthermore, the possibility to expand NK cells from lymphocyte-enriched cell fractions derived from PBMC by elutriation rather than utilizing isolated $CD56^+$ cells as the starting cell population was determined. In addition, cytolytic allogeneic and autologous activity by direct cytotoxicity as well as antibody-mediated cellular cytotoxicity and NK specific receptor-ligand interactions that mediate target cell killing were defined.

We confirmed that large quantities of cytotoxic NK cells can be expanded from PBMC in the presence of K562 cells expressing membrane-bound IL-15 and 4-1BBLigand from normal individuals and patients with various solid tumors. *Ex vivo* expansion tended to alter the balance of NK cell receptor expression towards those that activate and mediate cytotoxicity. This activity resulted in cytotoxicity against various allogeneic tumor targets and more importantly, against autologous-derived gastric tumor targets. Blocking studies identified multiple activating receptor-ligand interactions that would be predicted to mediate NK cell cytotoxicity. Moreover, these activating receptor-ligand interactions were operative in antibody-dependent cellular cytotoxicity (ADCC) in an allogeneic and autologous setting. Importantly, as a mean for future clinical translation, GMP compliant cytolytic NK cells could efficiently be expanded from lymphocyte-enriched cell fractions obtained from PBMC by counter current elutriation.

Our studies demonstrate that human NK cells acquire cytolytic activity against autologous gastric tumor cells after *ex vivo* expansion and suggest a therapeutic potential for autologous expanded NK cells, both directly and in combination with monoclonal antibodies in future cell-based immunotherapy.

Materials and methods

Cells and Cell Fractions

Human blood samples were purchased (BRT Laboratories, Baltimore, MD) and whole peripheral blood mononuclear cells (PBMC) were isolated using density-gradient centrifugation. Using leukapheresis products purchased from the same source, the constitutive cell populations were fractionated by continuous-counterflow elutriation following protocols established by the manufacturer of the Elutra cell separator (Elutra, Gambro BCT). This instrument uses continuous counter-flow elutriation technology to separate cells fractions based primarily by size and secondarily by specific gravity. In brief, the leukapheresis product was loaded via an inlet pump into a constantly rotating (2,400 rpm) elutriation chamber. Based on centrifuge speed and cell density, five elutriated cell fractions were collected. PBMC and various elutriated cell fractions were viably frozen in RPMI-1640 (Invitrogen Corp., Grand Island, NY) supplemented with 20% human AB serum (Gemini Bio-Products, Woodland, CA) and 10% Dimethylsulfoxide (Sigma, St. Louis, MO) using an automated cell freezer (Gordinier Electronics, Roseville, MI) and stored in the vapor phase of liquid nitrogen until used.

The myeloid cell line K562, prostate cancer cell lines LNCaP, PC-3 and DU-145 and breast cancer cell line MCF-7 were available in our laboratory. The lung cancer cell line H358 was kindly provided by Dr. S. Ostrand-Rosenberg (Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, MD, USA) and the Head and Neck cancer cell line TU-167 was kindly provided by Dr. S. Strome (Department of Otorhinolaryngology-Head and Neck Surgery, University of Maryland, Baltimore, MD, USA).

With patient consent and under approval of the Institutional Review Board, peripheral blood mononuclear cells were obtained from two patients with gastric cancer undergoing treatment at the Tokyo Clinic and Research Institute, Tokyo, Japan. Cell lines (tumor 1 and tumor 2) were established from biopsies of metastatic gastric tumor lesions from the respective patients. All tumor cell lines were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum, 1% P/S and 1% Glutamax-1 (cRPMI).

Ex vivo NK cell expansion

NK cells were expanded from PBMC as previously described with some minor modifications (12). In brief, PBMC (1.5 x 10^6) were incubated with irradiated (14,000 rad) K562-mbIL15-4-1BBL cells (10^6) in a 24-well tissue culture plate in the presence of 200 IU/ml human IL-2 (R&D Systems Inc) in cRPMI. Half of the culture medium was replaced every 2-3 days with fresh culture medium for the first 6 days. After 6 days of expansion, cells were harvested, washed, counted and re-cultured at a starting cell density of $1x10^5$-$3x10^5$/ml in T-25 or T-75 culture flasks in cRPMI supplemented with IL-2. Cells were expanded for an additional 8 days. Additional cRPMI was added to the flasks if necessary based on cell density.

Flow Cytometry

Cell surface expression was determined before and after 14 days of cell expansion by staining with directly conjugated mouse anti-human antibodies (mAbs) against CD3, CD56, αβTCR, γδTCR, HLA class I, HLA-DR, Fas, Fas-ligand, KLRD1, NKG2a, KIR3DL1, ILT2, CD62L, KIR3DL2/3, NKG2d, DNAM-1, NKp46, NKp44 and NKp30 (BD Biosciences). Gates were set around NK cells which were defined as $CD3^-CD56^+$ cells. Surface expression of NK cell ligands was determined on both autologous gastric tumor cell lines and included directly conjugated mouse anti-human nectin-2, PVR, MIC A/B, Fas, HLA class I, HLA class II, HLA-G and purified mouse anti-human HLA-E, ULPB-1, ULBP-2 and ULBP-3. For EGFR-mediated ADCC, gastric tumors were stained with mouse anti-human EGFR mAb. Mouse IgGs were used as isotype controls and purified mAbs were secondarily stained with FITC labeled goat anti-mouse mAb. A minimum of 10,000 events were acquired using a BD™ LSR II flow cytometer. Data was analyzed with BD™ FACS DIVA Software.

Cytotoxicity assays

Cytolytic NK cell activity was measured by 4 hour chromium 51 (^{51}Cr)-release assays as previously described (19). K562 cells were included in all cytotoxicity assays to assess overall cytotoxicity performance (data not shown). Expanded day 14 cells were purified into separate populations of NK cells ($CD3^-CD56^+$) and NKT/T ($CD3^+CD56^+$/$CD3^+CD56^-$) cells using MACS human CD3 microbeads and non-expanded NK cells were purified from PBMC using a MACS human NK cell isolation kit. (Miltenyi Biotec Inc). Cell purity was determined to be >92% and 95% respectively. To determine ADCC, 10μg/ml human IgG1 (huIgG1, Sigma-Aldrich Corp, St. Louis, MO, USA) or Cetuximab (University of Maryland Marlene and Stewart Greenebaum Cancer Center Pharmacy) was added to defined

wells. Purified mouse IgG1, mouse anti-DNAM-1, NKp46, NKp44, NKp30 or all four together (all at 10μg/ml) were added to defined wells during 4 hours of cytotoxicity in order to assess specific activating NK cell receptor-tumor ligand interactions. Reduction in cytotoxicity was calculated based on percentage cytotoxicity in the presence of indicated blocking mAb(s) versus percentage cytotoxicity in the presence of mouse control mAb. The % reduction in ADCC was calculated with percentage cytotoxicity in the presence of human IgG1 set at 100%. To minimize changes that may occur when cell lines are established from primary tumors, the gastric cell lines used in these studies were cultured for less than 10 passages after isolation from the primary tumor tissue.

Statistics

Paired two-tailed Student's t tests were used to calculate p values. $P<0.05$ was considered to be significant.

Results

Cytotoxic NK cells are efficiently expanded from PBMC from normal individuals and patients with various solid tumors without the need of primary enrichment protocols.

To achieve large-scale expansion of human NK cells, PBMC were co-cultured in a 1 to 1.5 ratio with lethally irradiated K562 cells expressing membrane-bound IL-15 and 4-1BBLigand (K562-mbIL15-4-1BBL) in culture media containing 200 units IL2 /ml. After 14 days of culture, NK cells ($CD56^+CD3^-$ as defined by flow cytometry) expanded greater than 2 orders of magnitude from PBMC (mean 165 fold; range 4-567 fold, n=6) and cell products became significantly enriched in NK cells (day 0 with mean 7%, range 3.2%-12.6% versus day 14 with mean 45.6%, range 7.4%-76.4%; P=0.0140). At the same time, NKT cells ($CD56^+CD3^+$ as defined by flow cytometry) expanded at an average of 57 fold (range 7-234), although no significant enrichment (day 0 with mean 3.8%, range 0.8%-8.1% versus day 14 with mean 11.4%, range 2.3%-17.9%; P=0.1907) was observed. In contrast, a significant decrease in T cells ($CD3^+$ as defined by flow cytometry) was noted after 14 days of expansion (day 0 with mean 54.5%, range 39.9%-71.2% versus day 14 with mean 30.0%, range 4.2%-58.4%; P=0.0436) with an absolute expansion of 7 fold (range 2-19). The distribution of NK cells and NKT cells in PBMC after expansion is shown in Fig 1A. Importantly, *ex vivo* expanded NK cells from healthy donor PBMC efficiently lysed allogeneic breast-and prostate-derived tumor targets but not allogeneic or autologous PBMC (Figure 1B). We did observe that cytotoxicity was associated with overall expansion efficiency. Specifically, the

one donor whose cells expanded 4 fold after 14 days of culture demonstrated an average of 11.7% cytotoxicity (effector to target ratio 1:10) against K562 cells, whereas donors who expanded an average of 202 fold (range 34-576; n=4) possessed an average of 59.8% cytotoxicity (range 56.0%-65.9%; n=4) against K562 cells (data not shown).

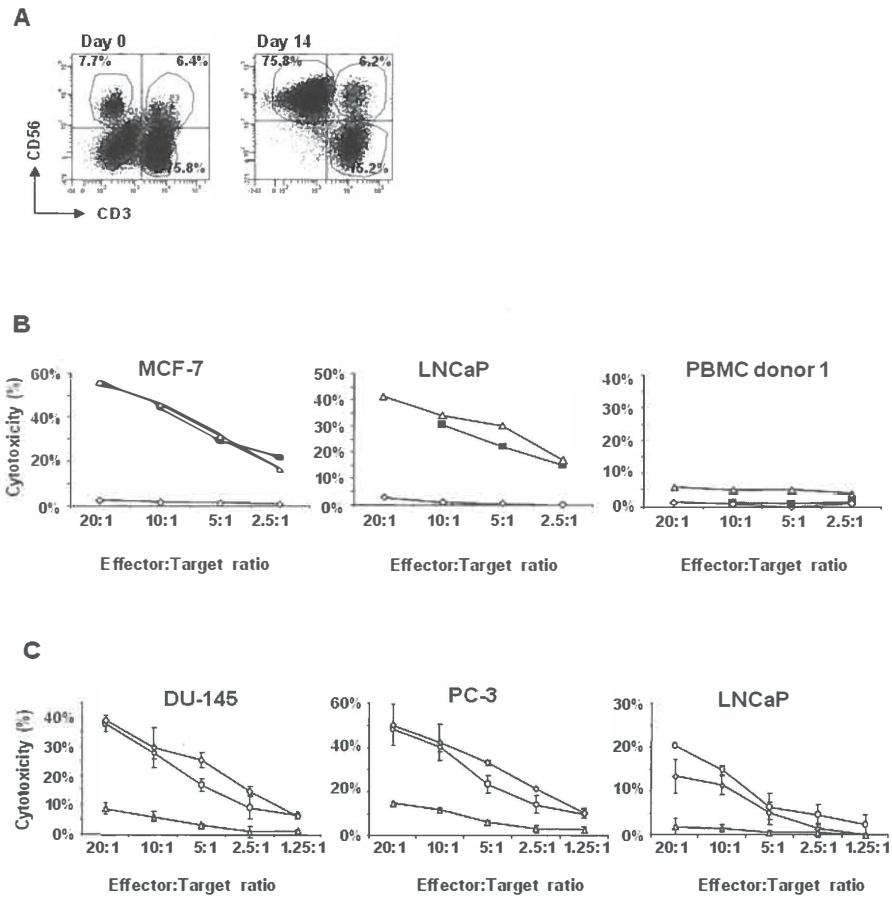


Figure 1. Cytolytic NK cells are efficiently expanded from PBMC. In the presence of K562-IL15-4-1BBL **(A)** expanded cells become significantly enriched (P=0.0307) in NK cells (defined by $CD56^{+}CD3^{-}$ cells) after 14 days of culture. Expanded cells were evaluated for cytolytic activity using 4 hour ^{51}Cr release assays. *Ex vivo* expanded cells from PBMC (■ donor 1 and Δ donor 2), but not freshly purified non-expanded NK cells (◊), efficiently lysed allogeneic tumor cell lines derived from breast (MCF-7) and prostate (LNCaP) cancers but not allogeneic or autologous PBMC derived from donor 1 **(B)**. The mean percentage cytotoxicity is shown from triplicate wells. Error bars represent the SD. Lytic activity is likely mediated by NK cells in the expanded cell population (○) since separation in individual populations of NK cells (◊) and NKT/T cells (Δ) resulted in allogeneic cytolytic activity of the expanded cell population and the purified NK cell population. Little lytic activity was observed in the presence of NKT/T cells alone **(C)**. The mean percentage cytotoxicity is shown from triplicate wells from one representative experiment. Error bars represent the SD. Experiment shown represents one of three individual experiments with three different donors.

Based on CD3 and/or CD56 phenotype, the majority of cells in the expanded cell products represented NK cells while a much smaller proportion represented NKT and T cells (Table 1). To determine if both the NK cells and NKT/T cells mediated cytolytic activity, the two populations were isolated by immunomagnetic bead selection and killing assays against prostate-derived tumor cell targets were performed. Cytolytic activity was mediated by NK cells and not NKT cells (Fig. 1C). Interestingly, little to no killing was observed with the NKT/T cell population even though a subpopulation of the T cells was confirmed to be γδ-TCR$^+$ by flow cytometry (data not shown). Although γδ-TCR$^+$ T cells are reported to have lytic activity against allogeneic tumor cells, they first require *in vitro* activation with isopentenyl pyrophosphate (IPP) and IL-2 (20). Studies are underway to determine if addition of IPP will expand a cytolytic γδ-TCR$^+$ population.

The capacity of K562-mb15-41BBL to stimulate expansion of NK cells from peripheral blood of healthy individuals and children with leukemia in remission was previously demonstrated (12,17). However, there is little information in reference to expand NK cells from PBMC derived from patients with solid tumors. With informed consent, NK cells were expanded from PBMC derived from patients with various solid tumors, including gastric cancer, lung cancer, colon cancer and hepatocellular cancer. After 14 days of culture, cell products became significantly enriched in NK cells (day 0 with mean 23.5%; range 5%-46% versus day 14 with mean 80%; range 60%-95%, n=6, P=0.0001 data not shown). Expansion efficiency was comparable between PBMC derived from solid tumor patients versus healthy donor PBMC (mean 316 fold; range 1-1795 with n=6 versus mean 165 fold; range 4-567 with n=6, P=0.6685). These data suggest that NK cells are efficiently expanded from PBMC from normal individuals and more importantly, from patients with various solid tumors without the need of primary enrichment protocols.

NK cell expansion turns the receptor balance towards activation and results in autologous gastric tumor cell lysis.

Human NK cells maintain self tolerance by the expression of at least one inhibitory receptor specific for autologous HLA class I which prevents cytotoxicity against autologous cells (21). To establish cytotoxicity against autologous target cells, inhibitory signals must be overcome, either by (i) down-regulation of inhibitory ligands on the tumor cell, (ii) enhanced expression of activating receptors on NK cells, (iii) expression of ligands on the tumor target that activate the NK cell or (iv) a combination of thereof. Since NK cell activation is affected

Table 1 Cell phenotype and fold expansion after 14 days of expansion

Donor	$CD3^-CD56^+$ NK cells population (%)	$CD3^-CD56^+$ NK cells expansion (fold)	$CD3^+CD56^+$ NKT cells population (%)	$CD3^+CD56^+$ NKT cells expansion (fold)	$CD3^+CD56^-$ T cells population (%)	$CD3^+CD56^-$ T cells expansion (fold)
1	7.4	4	17.9	31	58.4	4
2	61.7	140	4.2	26	21.2	9
3	68.5	61	3.1	7	23.1	4
4	76.5	183	2.3	12	4.2	2
5	35.6	576	37.2	234	22.1	19
6	23.9	34	3.8	33	51.2	7
mean:	45.6	165	11.4	57	30.0	7
range:	7.4-76.5	4-576	2.3-17.9	7-234	4.2-58.4	2-19

by cytokines such as IL-2 and IL-15 (22), we sought to determine if NK cells expanded from PBMC were phenotypically different from non-expanded NK cells (Table 2). In expanded NK cells from normal individuals, no significant change was observed in inhibitory receptors KIR3DL1 (P=0.1526), KIR3DL2/3 (P=0.7858) and the activating receptor NKG2D (P=0.1074). In contrast, activating receptors DNAM-1 (P=0.0061), NKp46 (P=0.0161), NKp44 (P=0.0039) and NKp30 (P=0.0131) were significantly increased in expression after 14 days of expansion. Interestingly, KLRD1 (P=0.0012) and NKG2A (P=0.0118), which both form a complex with the inhibitory non-classical HLA class I ligand HLA-E (23,24) were also significantly increased after expansion. Importantly, expression of the inhibitory receptor ILT2 (P=0.0142) which recognizes multiple HLA class I alleles, including non-classical HLA class I, HLA-G (25), was significantly decreased after expansion.

In order to define the ability of expanded NK cells derived from patients with solid tumors, to kill their autologous tumors, tumor cell lines were established from tumor biopsies from two metastatic gastric cancer patients undergoing immunotherapy at the Tokyo Clinic and Research Institute, Tokyo, Japan. Of note, the expression of inhibitory and activating receptors on expanded NK cells from the gastric cancer donors were generally not different from expression on expanded NK cells from normal donors (table 2). Since autologous NK cell cytotoxicity is the net result of engagement of activating NK cell receptors with activating target cell ligands, the two gastric tumor cell lines were first phenotypically characterized for expression of ligands (Table 3) that are known to engage the NK cell receptors identified in table 2.

Table 2 Phenotypic changes on human NK cells after 14 days of expansion

	healthy donor (n=6)					patient 1		patient 2	
	day 0		day 14			day		day	
	mean (%)	range (%)	mean (%)	range (%)	P-value[a,b]	0 (%)	14 (%)	0 (%)	14 (%)
activating receptors									
DNAM-1	83	72-90	94	89-97	0.0335 (↑)	90	97	37	90
NKG2D	83	51-98	96	93-99	0.1074	30	94	87	98
NKp46	68	27-91	87	64-97	0.0161 (↑)	52	95	19	70
NKp44	3	2-5	59	16-93	0.0039 (↑)	0.3	29	0.4	15
NKp30	52	11-93	82	67-97	0.0131 (↑)	7	63	15	70
inhibitory receptors									
KLRD1	68	56-82	92	86-95	0.0012(↑)	ND	98	49	95
NKG2A	46	14-67	68	34-89	0.0118 (↑)	ND	84	7	8
KIRDL1	22	10-37	29	17-38	0.1526	ND	21	5	3
KIR3DL2/3	28	9-48	29	14-44	0.7858	ND	35	88	96
LIR1	22	13-37	6	3-9	0.0142 (↓)	ND	18	70	44

[a]Significant differences (P<0.05) are indicated in bold
[b]Arrows indicate significant increase (↑) or significant decrease (↓)
DNAM-1 indicates DNAX accessory molecule-1; NKG2D, natural killer group 2 member D; KLRD1, killer cell lectin-like receptor subfamily D member 1; NKG2A, natural killer group 2 member A; KIRDL1, killer cell inhibitory receptor DL1; KIR3DL2/3, killer cell inhibitory receptor 3 DL2/3; LIR1, leukocyte Ig-like receptor 1; ND, not determined

While the ligands for human NKp46, NKp44 and NKp30 are to be defined, both patient cell lines expressed high levels of the inhibitory ligands HLA class I (75% and 67%, respectively) and HLA-G (42% and 57%, respectively) and relatively small amounts of the activation ligands MHC class I chain-related (MIC) A/B (2% and 1%, respectively), UL16 binding protein (ULBP)-1 (both 3%), ULBP-3 (both 3%) and polio-virus receptor (PVR; 8% and 9%, respectively). Importantly, both cell lines expressed the activating ligand nectin-2 (both 92%; specific for DNAM-1) which prompted us to evaluate both cell lines for their sensitivity against autologous NK cells. Subsequent 4 hour chromium-release (^{51}Cr-release) assays confirmed that gastric tumor cells derived from both patients were killed by autologous expanded NK cells (Figure 2A) and not by resting (non-expanded) NK cells from patient 2. Unfortunately, insufficient numbers of PBMC from patient 1 were available to isolate and test resting NK cells. Since expanded NK cells significantly up- regulated DNAM-1, NKp46, NKp44 and NKp30, we performed blocking studies in order to evaluate the importance of these activating receptor-ligand interactions in autologous tumor cell recognition (Figure 2B).

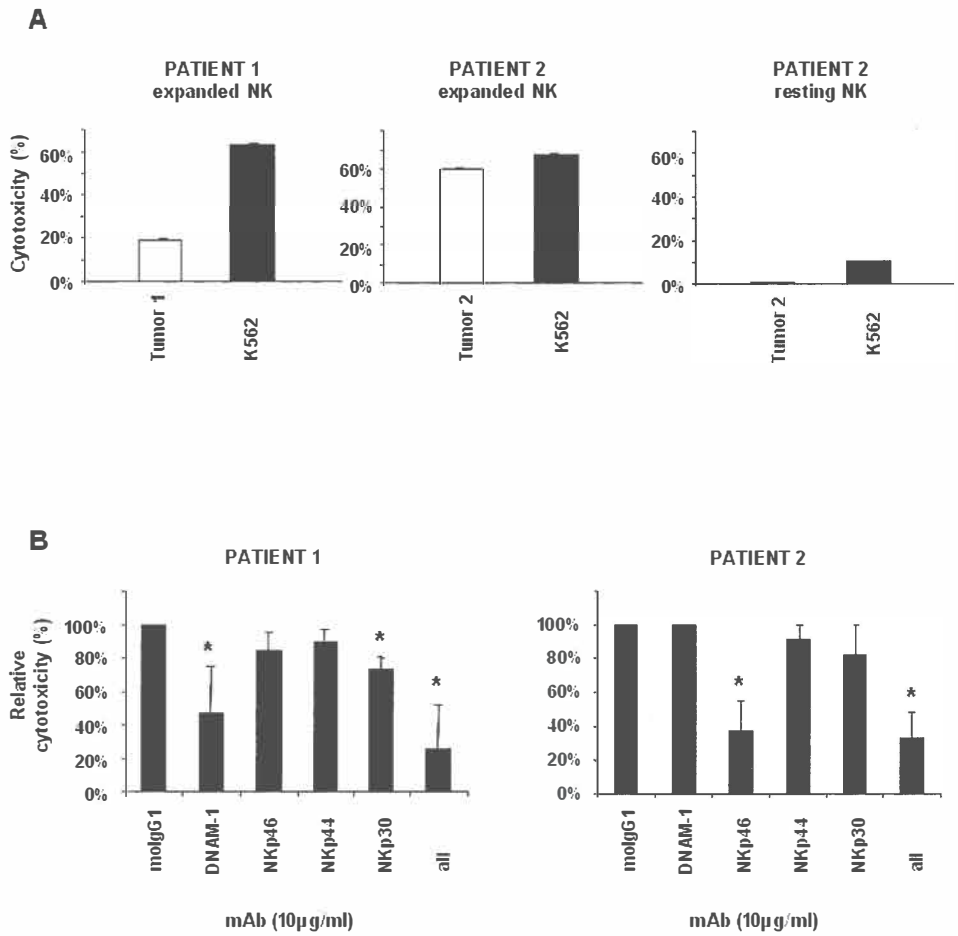


Figure 2. *Ex vivo* expanded NK cells recognize autologous gastric tumor cells through different activating receptor-ligand interactions. PBMC from two gastric cancer patients were *ex vivo* expanded for 14 days and then tested for cytolytic activity against autologous gastric tumor cells in 4 hour ^{51}Cr release assays. **(A)** *Ex vivo* expanded cells from both patients (patient 1 and patient 2), but not freshly purified non-expanded NK cells from patient 2, efficiently lysed autologous gastric tumor cells (effector to target ratio 20:1). To evaluate the impact of activating receptor-ligand interactions on autologous tumor cell lysis indicated blocking antibodies (10µg/ml) were added during 4 hours of incubation. **(B)** Cytotoxicity was reduced in the presence of DNAM-1 (P=0.0309) and NKp30 (P=0.0056) for patient 1 and in the presence of NKp46 (P=0.0003) for patient 2. In both patients autologous cytolytic activity was abrogated in the presence of all four blocking antibodies with P=0.0111 and P=0.0001, respectively. Statistical analysis is based on triplicate wells of four (patient 1) and two (patient 2) experiments performed, respectively. Error bars represent the SD. * P<0.05. MoIgG1 indicates mouse IgG1; DNAM-1, DNAX accessory molecule-1.

As expected, autologous lytic activity was significantly reduced (P=0.0111 for patient 1 and P=0.0001 for patient 2) when activating receptor-ligand interactions were interrupted by all four blocking mAbs. Specifically, lytic activity of autologous NK cells from patient 1 was significantly reduced in the presence of

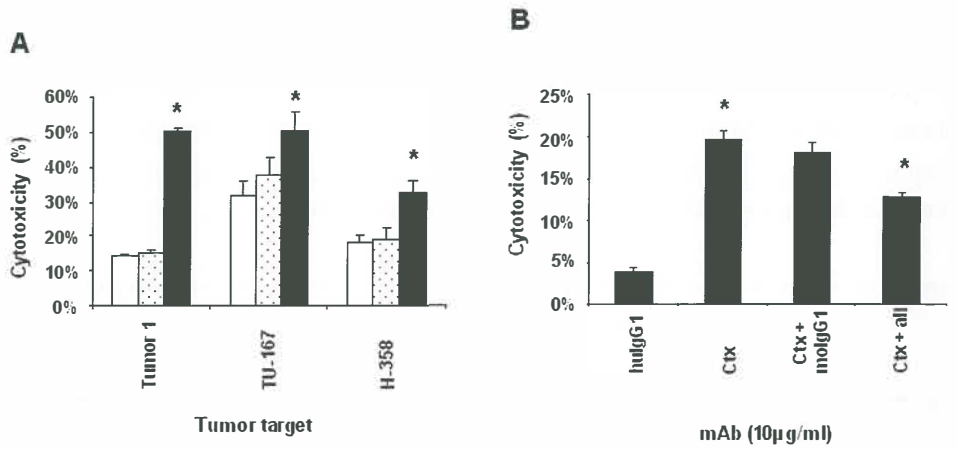


Figure 3. Cetuximab significantly enhances cytolytic activity and ADCC is negatively affected by inhibition of activating receptor-ligand interactions. *Ex vivo* expanded cells from cancer patient 1 were evaluated for their ability to mediate ADCC against autologous (patient 1) and allogeneic (TU-167 and H-358) EGFR expressing cancer cells. **(A)** Cytolytic activity of *ex vivo* expanded cells was enhanced in the presence of Cetuximab (10µg/ml, black bar) but not in the presence of control human IgG1 (10µg/ml; dotted bar) or media alone (white bar). The mean percentage cytotoxicity is shown from triplicate wells from one representative experiment. Error bars represent the SD. Experiment shown represents one of two individual experiments. **(B)** The addition of blocking antibodies (10µg/ml) against DNAM-1, NKp46, NKp44 and NKp30 (= all) significantly reduced (P=0.0176) Cetuximab-mediated ADCC. Statistical analysis is based on three experiments performed. Error bars represent the SD. * P<0.05. HuIgG1 indicates human IgG1, Ctx; Cetuximab and moIgG1; mouse IgG1.

mAb against DNAM-1 (P=0.0309) or NKp30 (P=0.0056) while lytic activity of autologous NK cells from patient 2 was only affected in the presence of mAb against NKp46 (P=0.003).

Ex vivo expanded NK cells are capable of autologous and allogeneic target cell lysis by antibody-mediated cellular cytotoxicity

Over many years, it has been postulated that eradication of human tumors may best be accomplished by combining cancer treatment modalities (26,27). Monoclonal antibodies that react with cell surface structures expressed on cancer cells represent the most successful cancer immunotherapy to date. It is quite clear that their mechanism of action is, at least, partially due to NK cell-mediated ADCC (28). Since expanded NK cells expressed high levels of CD16 (data not shown), an Fc receptor that mediates ADCC, we sought to determine if lytic activity against the gastric tumor cells could be enhanced in the presence of Cetuximab (Erbitux®), a chimeric monoclonal antibody that reacts with the EGFR receptor and is used to treat patients with a variety of solid tumors (29). Both gastric tumor cell lines were screened for EGFR and only one of the two patient tumor cell lines (patient 1) expressed EGFR (table 3).

Subsequent ^{51}Cr-release assays confirmed that allogeneic and autologous cytolytic activity is greatly enhanced in the presence of chimeric anti-EGFR mAb but not in the presence of human IgG1 control antibody (Figure 3A). As expected, the enhancement in cytotoxicity was far more dramatic in the autologous setting if compared to the allogeneic setting since allogeneic tumor cells do not maintain self tolerance through specific inhibitory receptor-ligand interactions.

Importantly, the expression of activating receptors on the *ex vivo* expanded NK cells positively affected overall cytotoxic activity (Figure 3B) since blocking all four activating receptors on the NK cell surface decreased autologous cytotoxicity if compared with control mAb (P=0.0176 and P=0.1019, respectively). These data suggest that the combined strategy of adoptively transferred *ex vivo* expanded autologous NK cells with infusion of a mAb that is used for cancer immunotherapy may provide clinical benefit for the treatment of select human solid malignancies. To extend these observations, we are attempting to establish cell lines from other solid tumors where PBMC would be available to test NK expansion, direct cytotoxicity and ADCC capability.

Table 3 Characterization of NK cell ligands on gastric tumor cells

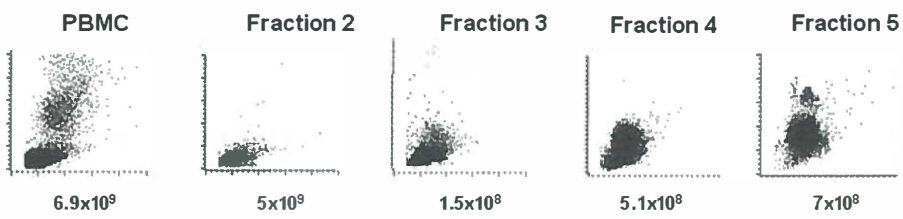
	patient 1 (N=2)		patient 2 (N=3)	
	mean (%)	range	mean (%)	range
inhibitory ligands				
HLA class I	75	71-80	67	30-97
HLA-E	1	0-1	2	1-3
HLA-G	42	28-56	57	30-82
activating ligands				
PVR	8	3-14	9	3-18
Nectin-2	92	87-98	92	87-97
MIC A/B	2	1-2	1	0-1
ULBP-1	3	2-4	3	2-4
ULBP-2	62	60-65	67	51-76
ULBP-3	3	3-4	3	2-4
other				
Fas	36	21-50	95	88-99
EGFR	95	93-98	18	7-29

PVR indicates polio-virus receptor; MIC A/B, MHC class I chain-related A/B; ULBP, UL binding protein; EGFR, epidermal growth factor receptor.

NK cells are efficiently expanded from lymphocyte-enriched cell fractions obtained from PBMC by counter current elutriation

A GMP compliant system has successfully been established for the enrichment of monocytes from PBMC using an Elutra cell separator. In this closed system, PBMC are fractionated by centrifugal elutriation and five cell fractions are obtained. In general, these fractions consist of platelets (fraction 1), erythrocytes mixed with lymphocytes (fraction 2), lymphocytes (fraction 3), lymphocytes mixed with monocytes (fraction 4) and mainly monocytes (fraction 5) as demonstrated in figure 4 (n=11). Current clinical cellular therapy protocols use monocytes obtained from elutriated fraction 5 to generate dendritic cells for cancer immunotherapy while the cells from fractions 2, 3 and 4 are usually "archived" in liquid nitrogen. As a means to facilitate clinical translation, we explored the possibility of these GMP compliant cell fractions to serve in future NK cell-based immunotherapy studies. PBMC and separate elutriated cell fractions were expanded with the aforementioned expansion strategy. After 14 days of culture in the presence of K562-mbIL15-41BBL cells and exogenous IL-2, NK cells expanded greater than

A



B

CD3+	47+/-13	61+/-20	55+/-21	24+/-16	25+/-22
CD4+	17+/-14	39+/-9	36+/-13	47+/-23	32+/-28
CD8+	11+/-7	25+/-13	22+/-12	7+/-7	3+/-4
CD3+CD56+	6+/-7	5+/-6	15+/-11	4+/-7	3+/-6
CD3-CD56+	4+/-4	4+/-4	8+/-7	6+/-6	2+/-3
CD14+	5+/-6	0+/-0.4	9+/-6	56+/-19	80+/-12
CD19+	5+/-4	9+/-5	3+/-2	3+/-2	2+/-3

Figure 4. Distribution of lineage-specific phenotypic markers on PBMC and separate cell fractions obtained after counter current elutriation. PBMC and elutriated cell fractions were stained with various lineage-specific directly-conjugated antibodies and analyzed by flow cytometry. **(A)** Average number of cells and phenotypic distribution (%) expressing lineage-markers in elutriated cell fractions (n=11) **(B)**.

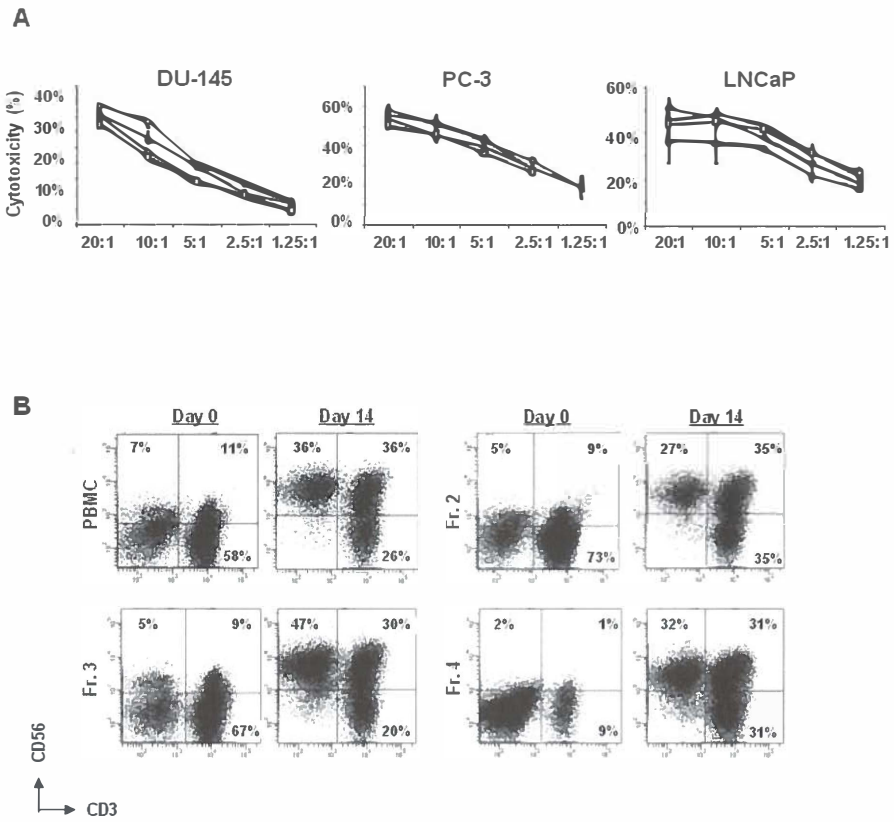


Figure 5. ***Ex vivo*** **expanded cells from elutriated cell fractions efficiently lyse allogeneic prostate cancer cells.** PBMC and elutriated fractions 2, 3 and 4 from the same healthy individual were expanded *ex vivo* in the presence of K562-mbIL15-41BBL and IL-2 for 14 days and then tested for *in vitro* cytolytic activity. Cytolytic activity was evaluated in 4 hour ^{51}Cr release assays against **(A)** prostate cancer (DU-145, PC-3 and LNCaP) cells. *Ex vivo* expanded cells from elutriated cell fractions 2 (◊), 3 (Δ) and 4 (□) lysed prostate cancer cells in a similar fashion as *ex vivo* expanded cells from PBMC (○). **(B)** Elutriated cell fractions become enriched in NK cells (defined by $CD56^{+}CD3^{-}$ cells) after 14 days of culture regardless the cellular content of these fractions. The mean percentage cytotoxicity is shown from triplicate wells from one representative experiment. Bars represent the SD. Experiment shown represents one of four individual experiments.

two orders of magnitude from PBMC (mean 165 fold; range 4-567 fold with n=6, data not shown), elutriated cell fraction 2 (mean 209 fold; range 3-615 fold with n=3, data not shown), elutriated cell fraction 3 (mean 131 fold; range 4-339 fold with n=3, data not shown) and elutriated cell fraction 4 (mean 91 fold; range no expansion-358 fold with n=4, data not shown). Importantly, expanded cells from PBMC and separate elutriated cell fractions became significantly enriched in NK cells and lysed allogeneic prostate-derived tumor cell lines in a similar fashion (Figure 5A-B). Thus, these data show that large quantities of cytolytic NK cells can

be expanded from various elutriated cell fractions collected with the GMP compliant Elutra system.

Discussion

The use of NK cells as a cancer treatment modality in the absence of an allogeneic stem cell transplant requires that large quantities of NK cells are generated that kill the tumor cells directly or augment the cytotoxic effect of tumor directed monoclonal antibodies. The critical findings of the studies reported herein are that (i) the technology that was reported to successfully expand NK cells isolated from PBMC (12,17) was also effective in generating large numbers of NK cells from PBMC and various elutriated cell fractions without previous isolation of the $CD56^+$ cells, (ii) activation receptors and other cell surface structures that mediate target cell killing are increasingly expressed on the expanded NK cells, (iii) NK cells expanded from PBMC from patients with solid tumors kill allogeneic and autologous cancer cell lines by direct cytotoxicity and, importantly, mediate ADCC to autologous tumor cell targets in the presence of chimeric mAb and (iv) the interactions of activating receptors DNAM-1, NKp30 and NK-p46 with cognate ligands on the tumor target appear to mediate direct and ADCC mediated autologous cytotoxicity in gastric carcinoma.

For translation into the clinic it is important to observe that besides NK cells, relatively small numbers of NKT and T cells are expanded in this system. These cell populations may mediate GvHD when infused together with NK cells in adoptive allogeneic immunotherapy protocols. GvHD is a serious, potentially life-threatening, condition resulting from transplanted or infused allogeneic donor cell recognition of the recipients' tissues as non-self, and is predominantly mediated by $CD3^+$ T cells (30). These cells are often depleted to prevent GvHD, as could be accomplished with the cells expanded by the presented protocol. Depletion of T cells from the NK cell product before administration to the host is likely to be less critical in an autologous setting.

An important observation in our studies was that the expanded NK cells did not kill autologous and allogeneic PBMC, an indication that despite the increase in surface expression of activating receptors on the NK cells, the inhibitory ligands expressed on normal PBMC were dominant and able to control cytolytic activity against non-malignant cells. This is further illustrated by the fact that both gastric tumor cell lines were susceptible to autologous cytotoxicity despite the expression of high levels of inhibitory classical and non-classical HLA class I molecules. These data

suggest that, in certain circumstances, activating receptor-ligand recognition may override receptor-ligand interactions that inhibit NK activity. Emerging data indicates that important triggers in this interaction are surface structures (ligands) that are expressed on cells that have undergone malignant transformation. In addition, it is well recognized that HLA class I expression, the major NK cell inhibitory structure, is often down-regulated in many solid tumors. In the case of autologous NK cell cytotoxicity against PBMC, inhibitory signals still predominated over activating signals, since no cytotoxicity of NK cells against autologous or allogeneic PBMC was observed. Our results indicate that the NK cells expanded and activated by the methods described do not recognize and kill non-transformed cells.

While significantly higher levels of the inhibitory CD94/NKG2A complex were expressed after *ex vivo* cell expansion, it did not affect the potential of autologous gastric tumor cell recognition. The CD94/NKG2A complex is reported to directly inhibit NK cell cytotoxicity through recognition of HLA-E (31). Although both autologous gastric tumor cell lines did not express HLA-E (Table 3), we cannot rule out that autologous tumor cell recognition will not be affected in HLA-E expressing tumors.

The ligands for natural cytotoxicity receptors NKp30, NKp44 and NKp46 are currently unknown. However, we postulate that at least NKp46 and NKp30 may be involved in autologous gastric tumor cell recognition since lytic activity was abrogated in the presence of blocking antibody against these receptors. Since no significant change was observed in NKG2D expression on expanded NK cells, we did not directly test the involvement of this activating receptor in autologous gastric tumor cell cytotoxicity. The fact that autologous cytotoxicity was not completely inhibited by a combination of anti-DNAM-1, NKp46, NKp44 and NKp30 may indicate that NKG2D or other unidentified receptors may also be involved. Importantly, the interaction between NK cell receptors and their ligands has recently been shown to abrogate NK cell mediated cytotoxicity of human and mouse melanoma cell lines (32).

Of note, both tumor cell lines also expressed high levels of Fas which is recognized to establish cell death upon interaction with its ligand, Fas-ligand (33). In order to test the possibility of target cell-induced killing of the expanded NK cells, all NK cells were evaluated for Fas and Fas-ligand expression before and after *ex vivo*

expansion. Although expanded NK cells up-regulated high levels of Fas, they did not express Fas-ligand (data not shown).

It has been suggested that in order to overcome self tolerance, multiple activating receptor-ligand interactions should be engaged (31). Indeed, multiple activating interactions appear to be involved in autologous cytotoxicity of tumor cells derived from patient 1 when the inhibition of cytotoxicity, in the presence of all 4 antibodies, is compared with DNAM-1 or NKp30 alone (P=0.0356 and P=0.0165, respectively). In contrast, no significant additional decline was observed in autologous cytotoxicity for patient 2 when cytolytic activity of all four activating receptors was compared to NKp46 alone (P=0.7359). We postulate that these data reflect variation in expression of receptor-ligand combinations in human that are known to be operative in the control of NK cell cytotoxic activity. These variations include HLA and KIR polymorphism as well as tumor type and tumor origin (e.g. primary versus metastatic tumor cells). This is illustrated in a recent report on studies in patients with multiple myeloma (34) were the investigators demonstrated no specific association of autologous NK cell cytotoxicity with a single activating NK cell receptor. In fact, autologous cytotoxic effects were more likely mediated by several activating NK cell receptors which is also in agreement with a previous report (35) demonstrating that natural cytotoxicity of resting NK cells requires co-activation by more than a single receptor.

While currently elutriated cell fraction 5 is used for monocyte and dendritic cell-based immunotherapy therapy protocols (36,37), we demonstrated that cytolytic NK cells can be expanded from elutriated cell fractions 2, 3 and 4 regardless of the cellular content of these fractions. However, since NK cell expansion from fraction 4 failed in two out of four experiments, while expansion from PBMC and elutriated cell fractions 2 and 3 was highly successful, and considering the relative high amount of erythrocytes in fraction 2, it may be best to primarily utilize fraction 3 in NK cell expansion protocols. Of note, variability in expansion rates between donors is observed and requires further testing to determine the extent of this variation in the general population. Overall, these data provide a foundation for the large-scale generation of cytolytic NK cells from elutriated cell fractions, which could be employed alone or in combination with other cellular components such as dendritic cells for application in cellular therapy of cancer.

In summary, the large numbers of cytotoxic NK cells generated by this *ex vivo* expansion protocol provides the numbers of NK cells that will probably be required

to be effective in the case of a large tumor burden. The ability of the expanded cells to mediate ADCC offers the possibility that their effect may be amplified if given in conjunction with a cancer cell directed mAb. An important issue to address is the ability of adoptively transferred NK cells to home and infiltrate into solid tumor tissue. Although the expanded NK cells only expressed small amounts of CD62L (data not shown) which is associated with homing into secondary tissue, we postulate that trafficking to the tumor micro-environment may be enhanced by opsonizing tumor cells with chimeric antibody. Clinical studies are needed to confirm this hypothesis, as well as to establish the therapeutic benefit of infusion of large number of *ex vivo* expanded autologous NK cells.

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FcγRIIIa polymorphisms and cetuximab-induced cytotoxicity in squamous cell carcinoma of the head and neck

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Abstract

The interaction of Fc fragments of antibodies with the Fcγ receptors is an essential checkpoint in antibody-dependent cellular cytotoxicity (ADCC). Specific polymorphisms at position 158 enhance FcγRIIIa affinity for IgG1 and are associated with improved clinical outcome in lymphoma patients treated with IgG1 anti-CD20 antibody. The role of ADCC in the therapeutic effects of the α-epidermal growth factor receptor (EGFR) mAb, cetuximab, in patients with squamous cell carcinoma of the head and neck (SCCHN) is poorly defined. We employed three SCCHN cell lines to test two hypotheses: (i) SCCHN is susceptible to cetuximab-mediated ADCC and (ii) efficacy of ADCC is associated with polymorphisms at position 158 of FcγRIIIa. FcγRIIIa-158 polymorphisms were determined for healthy donors, and their purified NK cells were used as effector cells against three SCCHN cell lines in ADCC assays. Cytotoxicity levels were compared for each polymorphism. Proliferation and cell cycle assays were performed to examine the direct effects of cetuximab. Our results indicate that SCCHN is susceptible to cetuximab-mediated ADCC *in vitro*. NK cytotoxic efficiency correlates with donor 158-polymorphisms in FcγRIIIa. Overall cytotoxicity was greatest for individuals having a single *V* allele when compared to homozygous *F/F* individuals; the cumulative percent cytotoxicity for each polymorphism among the cell lines was 58.2% for *V/V*, 50.6% for *V/F*, and 26.1% for *F/F*. Additionally, the presence of a *V* allele correlated with superior natural cytotoxicity against NK sensitive targets. These data have both prognostic and therapeutic relevance and support the design of a prospective trial to determine the influence of FcγRIIIa polymorphisms on the clinical outcome of patients with SCCHN treated with α -EGFR mAbs.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) affects nearly 40,000 new patients in the United States annually and half a million worldwide, accounting for nearly 5% of all new solid tumors (18). Current SCCHN organ preservation options combine chemotherapy and radiation while newer approaches incorporate targeted therapeutics. Currently, the only approved targeted therapy for SCCHN is the epidermal growth factor receptor (EGFR) inhibitor, cetuximab (Erbitux®). Between 80-100% of SCCHNs over-express the EGF receptor, and the levels of over-expression are critical to overall tumor survival and associated with decreased treatment responses (1,15). Despite evidence that cetuximab in combination with radiotherapy improves outcome, its impact on both response rate and survival is incremental, with significant individual variability (4,6,7,11). One means to improve the therapeutic benefit of cetuximab is to define the population most likely to respond, through improved characterization of mode of action.

It is postulated that cetuximab's primary mechanism of action results from EGFR blockade. Cetuximab is an IgG1 α-EGFR chimeric mAb that blocks the binding of natural receptor-ligand interactions, preventing ligand-dependent homodimerization, intracellular autophosphorylation, and activation of intracellular cascades that control cellular proliferation, adhesion, angiogenesis and apoptosis (9,10,14). Binding of cetuximab to the EGFR leads to internalization and degradation of the antibody-receptor complex and down-regulation of EGFR expression (6). Because of the high propensity for over-expression of EGFR on SCCHN, cetuximab's ability to block EFGR is especially advantageous in head and neck cancer.

Another potential mechanism of action of cetuximab, which has remained largely unexplored to date, is antibody-dependent cellular cytotoxicity (ADCC). ADCC is increasingly recognized as an important component of the clinical efficacy seen in targeted antibody therapy for solid tumors (6,10,20). Growing experience with mAb targeted therapy in lymphoma patients demonstrates that select subpopulations experience superior clinical outcomes based upon FcγRIIIa polymorphisms known to influence ADCC. The most relevant polymorphisms regulating Fc:FcR interactions are phenylalanine (*F*) or valine (*V*) expression at position 158 of the Fc fragment (12,19). Indeed, Weng *et al* demonstrated that the clinical response to rituximab in patients with non-Hodgkin's lymphoma is predicted by the specific FcγRIIIa (CD16) polymorphism that they possess (32). Similarly, in another study involving lymphoma patients treated with rituximab,

individuals possessing the FcγRIIIa 158 *V/V* genotype experienced a better tumor response and survival (5). It is postulated that the higher affinity binding of FcγRIIIa containing a *V* allele enhances ADCC and improves clinical response.

In this study we show for the first time that cetuximab can mediate ADCC in SCCHN, and more importantly, that these effects are more pronounced in individuals with a *V* at position 158 on FcγRIIIa. Unexpectedly, we also found that the presence of a *V* allele in donor NK cells is associated with enhanced tumoricidal activity against the NK sensitive K562 cell line, suggesting a broader enhanced cytotoxic phenotype associated with the *V* allele. Our results support the design and implementation of a clinical trial correlating FcγRIIIa polymorphisms with outcomes in SCCHN patients treated with cetuximab.

Materials and methods

NK cell separation

Healthy human buffy coat in acid citrate dextrose anticoagulant was purchased from Biological Specialty Corporation (Colmar, PA). PBMC were isolated from the buffy coat using the Ficoll-Hypague centrifugation method. NK cells were then negatively selected using a MACS human NK cell isolation kit (Miltenyi Biotec, Auburn, CA).

Cell culture

SCCHN cell lines TU167 and TU159 were obtained from the MD Anderson Cancer Center, University of Texas, Houston, TX, USA. The cell line 012SCC was donated by Bert O'Malley from the University of Pennsylvania School of Medicine, Philadelphia, PA, USA. Cells were cultured in RPMI 1640 complete media containing 10% heat-inactivated fetal bovine serum (Atlanta Biologics, Lawrenceville, GA), 1% L-glutamine (Gibco, Carlsbad, CA), 1% Penicillin-Streptomycin (Gibco, Carlsbad, CA), and 1% HEPES buffer (Mediatech Inc., Herndon, VA).

Flow cytometry

SCCHN cell lines were stained with the FITC-conjugated rat α-human EGFR mAb (clone ICR10, Chemicon, Temecula, CA) and APC-labeled cetuximab. Cetuximab was obtained from the Marlene and Stewart Greenebaum Cancer Center Pharmacy, University of Maryland School of Medicine, Baltimore, MD, USA and conjugated by Invitrogen (Thousand Oaks, CA) with APC-fluorochrome. All SCCHN cell lines were also stained for α-human CD16 expression (BD Biosciences, San Jose,

CA). To evaluate NK purity after negative selection, isolated cells were stained with directly conjugated mouse α-human mAbs against CD56, CD11c, CD16, CD14 and CD3 (BD Biosciences, San Jose, CA). A minimum of 10,000 events were acquired using a BD™ LSR II flow cytometer and analyzed with BD™ FACS DIVA Software.

Determination of FcγRIIIa polymorphisms

Genomic DNA from human PBMC was obtained using the Qiagen DNA extraction kit (Qiagen, Valencia, CA) and stored at -20°C. The FcγRIIIa valine (*V*) or phenylalanine (*F*), at position 158 was determined by polymerase chain reaction (PCR), as described by Nieto *et al*, with modifications (26). Briefly, the PCR reaction was optimized using 250ng of DNA, 0.5mM dNTPs, 1 unit GoTaq polymerase (Promega, Madison, WI) and corresponding 1X buffer containing 1.5 mM $MgSO_4$ to a final volume of 50μL. For PCR amplification, samples were subjected to an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles at 94°C for 40 seconds, 52°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Amplified PCR samples were cleaned using phenol extraction and ethanol precipitation prior to restriction digestion. Two digestions per sample were performed to distinguish the FcγRIIIa polymorphisms. First, 10μL of the PCR product was incubated at 37°C overnight with 10 units of RsaI with the appropriate 1X buffer (Promega, Madison, WI) to a final volume of 20μL. For the second digestion, 10μL of the RsaI digested sample was subsequently incubated at 37°C overnight with 40 units of Eco130I with the appropriate 1X buffer (Fermentas, Hanover, MD) to a final volume of 30 μL. Both RsaI and RsaI/Eco130I digested products were separated on a 3% agarose gel with ethidium bromide and visualized under UV light.

Antibody dependent cellular mediated cytotoxicity

ADCC assays were performed using SCCHN cells as targets, and purified NK cells as effectors. Target cells were incubated with 150 μCi of chromium-51 (^{51}Cr) (Amersham, Piscataway, NJ) at 37°C for 1 hour, mixing thoroughly every 15 minutes, and washed twice with media. Cells were subsequently incubated with 10 μg/mL of cetuximab, 10 μg/mL of human IgG1 isotype, or media alone for another 30 minutes at 37°C, and washed twice with media to remove unbound antibody. Effector and target cells were plated at 50:1, 25:1, and 12.5:1 in 96 well plates and incubated overnight. Each assay was set up in triplicate wells. Cell lysis supernatant was collected and mixed with the Optiphase Supermix scintillation fluid (Perkin Elmer, Boston, MA) and counted in a MicroBeta 1450 scintillation

counter (Wallac, Turku, Finland). The NK sensitive K562 tumor cell line was used as a positive control for all ADCC experiments. Results were expressed as the percentage of specific lysis according to the following formula: (Experimental cpm-spontaneous cpm) x 100 / (maximum cpm-spontaneous cpm).

Thymidine proliferation assay

Growth inhibition of SCCHN in the presence of cetuximab was evaluated using thymidine assays. Each cell line was seeded at day 1 to a 96-well tissue culture plate at $0.1x10^4$ cells / well in 100μL RPMI complete media. For dose-dependent inhibition assays, cetuximab or IgG1 isotype controls were added to final concentrations of 10μg/mL, 0.1μg/mL, 0.01μg/mL, 0.001μg/mL and 0.0001μg/mL to the appropriate wells. For media only controls, 100 μL of media was added to the appropriate wells. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 48 hours. Thymidine was added on day 3 to a concentration of 1μCi /well, and plates were incubated for an additional 14-16 hours. Cells were embedded onto collection filters using the Packard Cell Harvester (Packard Biosciences, Shelton, CT). The filters were dried and counted in β-scintillation fluid using the MicroBeta 1450 scintillation counter (Wallac, Turku, Finland).

Cell cycle distribution

A BrdU Flow Kit (BD Pharmingen, San Jose, CA) with 7-AAD was utilized to determine the effect of cetuximab on SCCHN cell cycle and DNA content. Tumor cells were seeded in 6 well plates at $1.5x10^5$ cells / well. Cetuximab or isotype controls were introduced at 10μg/mL and incubated for 48 hours. Then the cells were pulsed with BrdU on day 3 for 4 hours at 37°C, and stained according to manufacturer's protocol.

Statistical methods

Statistical analyses were performed using the SAS statistical software (SAS version 9.1; SAS Institute, Cary, NC) and S-plus. Mean cytotoxicity was computed for each polymorphism according to the equation listed previously. Welch modified two-sample t-test (if the normality was not plausible), student t-test (a Q-Q plot was used to assess the correctness of normality assumption) and analysis of variance were used to determine statistically significant differences in mean cytotoxicity among cells bearing FcγRIIIa polymorphisms. Differences were considered to be significant at p below 0.05.

Results

FcγRIIIa polymorphism distribution

To establish the genotype of the FcγRIIIa for each donor, we modified an existing protocol to obtain a robust PCR amplification/restriction digest methodology that permitted the typing of each donor sample. We evaluated the frequency of polymorphisms at amino acid 158 from 45 samples, which included freshly obtained BOMC from 17 healthy donors that were used for ADCC studies and an additional 28 frozen donor PBMC samples. The frequency for *F* allele was calculated to be 0.70, and *V* allele to be 0.30. The combined distribution of polymorphic classes from our fresh and frozen donors was 51.1% for *F/F*, 37.8% for *V/F*, and 11.1% for *V/V*.

Cetuximab treatment of NK cells containing the FcγRIIIa *V* allele increases efficiency of ADCC against SCCHN

In order to develop a model system to study ADCC for SCCHN, we first characterized the EGFR expression on our tumor targets. We confirmed the EGFR over-expression for the three different tumor cell lines (TU167, TU159 and 012SCC) utilized in this study using both a monoclonal rat-derived α-human EGFR antibody and cetuximab (Figure 1). In order to rule out positive staining due to secondary Fc binding, each tumor cell line was stained for the expression of FcγRIIIa (CD16) using commercially available α-CD16 mAb. Our data indicate that none of the SCCHN cell lines expressed CD16 (data not shown).

We first employed TU167 to determine whether cetuximab mediates ADCC, and to define the impact of FcγRIIIa polymorphisms. In our initial study, we employed TU167 as target cells. We found that cetuximab-mediated ADCC is enhanced against this cell line in individuals possessing a *V* allele at all effector to target ratios (Figure 2). Combinatorial analysis of all ADCC experiments against TU167 revealed that donors with either *V/V* (64.0%) or *V/F* (47.5%) had superior cytotoxic activity against TU167 when compared to NK cells from *F/F* (22.5%) individuals at a 50:1 effector to target (E:T) ratio, (p= 0.04 and 0.004, respectively). This effect is not cell line specific as the advantage of having at least one *V* allele when compared to *F/F* was also seen for 012SCC, where individuals with at least one *V* allele (*V/V* and *V/F*) had a combined mean percent cytotoxicity of 65.0 % compared to 34.5% for those with *F/F* (p=0.0019) at 50:1 ratio. While we observed a similar trend in polymorphism-associated killing against TU159, differences were not statistically significant.

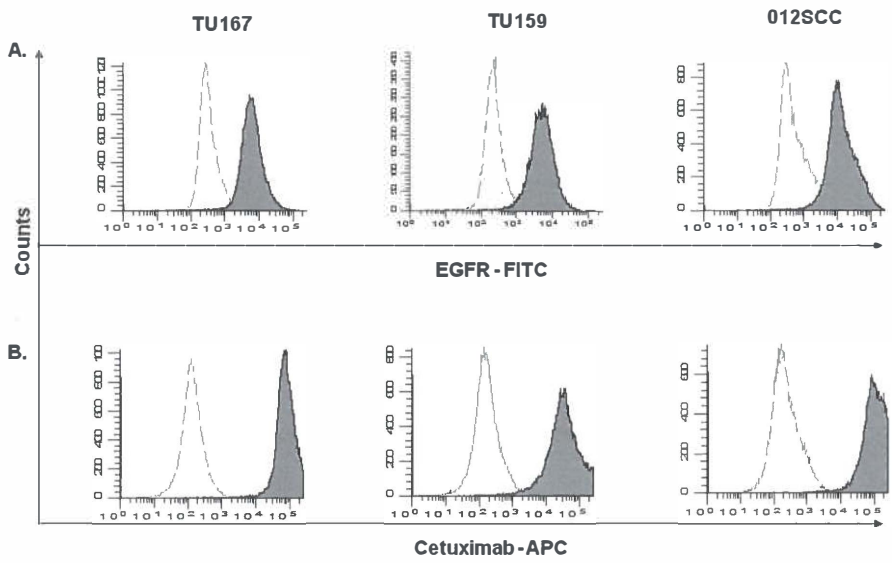

Figure 1. EGFR expression in SCCHN tumor cell lines. SCCHN cell lines TU167, TU59 and 012SCC were stained with α -EGFR mAb (clone ICR10) with an IgG2a isotype control (A) and Cetuximab with human IgG1 isotype control (B). Open histograms represent the isotype control. Filled histograms represent the mAb staining. All cell lines show a comparable shift for both α -EGFR and cetuximab mAb. EGFR indicates epidermal growth factor receptor.

This lack of significance was most likely due to the small sample size. Using an analysis of variance, we determined that there was a significant difference among the cumulative mean percent cytotoxicity for each polymorphism against all three SCCHN cell lines at a 50:1 effector to target ratio (58.2% for *V/V*, 50.6% for *V/F* and 26.1%for *F/F*; $p<0.001$). Cetuximab-treated SCCHN cell lines were subjected to higher levels of ADCC compared with cells treated with isotype controls or media alone. These data provide strong evidence for cetuximab-mediated ADCC in SCCHN, and demonstrate a direct correlation between cytotoxicity and the presence of a *V* allele at position 158 of FcγRIIIa (Table 1).

The presence of a *V* allele is associated with enhanced NK killing of NK sensitive targets

Our study and others have demonstrated that the presence of a *V* allele at position 158 enhances ADCC in both SCCHN and lymphoma (16,32,33). However, it is uncertain whether the impact of such polymorphisms correlates with other important intrinsic differences in NK cytotoxic potential.

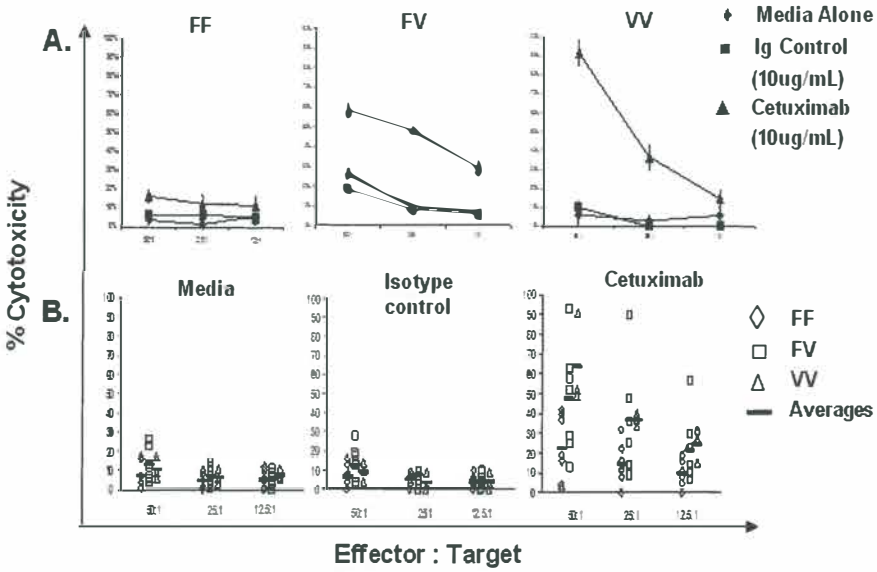

Figure 2. NK FcγRIIIa polymorphisms influencing cytotoxic activity on cetuximab-treated SCCHN TU167. NK cells purified from PBMC of healthy donors expressing either *F/F*, *V/F* or *V/V* alleles were tested for cytotoxicity against TU167 in a standard ^{51}Cr-release assay (A). Before addition of effector cells, TU167 target cells were pretreated with media alone (closed diamonds), human IgG1 isotype control at 10μg/ml (closed squares) or cetuximab 10μg/ml (closed triangles). Data are representative of independent experiments performed in triplicate wells. NK cells purified from PBMC of 17 individual donors *F/F* (open diamonds), *V/F* (open squares) and *V/V* (open triangles) alleles were tested for cytotoxicity against the TU167 SCCHN cell line using a standard ^{51}Cr-release assay (B). Before addition of effector cells, TU167 targets were pretreated with media alone, human IgG1 isotype control at 10μg/ml, or cetuximab at 10μg/ml. Donors with either FcγRIIIa *V/V* or FcγRIIIa *V/F* demonstrated significantly higher % cytotoxicity compared to FcγRIIIa *F/F*, when incubated with 10 μg/mL cetuximab (p= 0.04 and 0.004, respectively).

In order to test the hypothesis that NK cells with a *V* allele are associated with an enhanced cytotoxic potential unrelated to ADCC, we evaluated the cytotoxic ability of NK cells Interestingly, we observed that the presence of a *V* allele in donor NK cells was associated with significantly enhanced cytotoxicity against the NK sensitive K562 target (Figure 3). Using the Welch modified two-sample t-test, we determined that donors possessing FcγRIIIa *V/F* and *V/V* at 50:1, 25:1 and 12.5:1 have significantly higher cytotoxic activity than that of FcγRIIIa *F/F* donors (p=0.17, 0.044 and 0.043 respectively, CI=90%). These data suggest, for the first time, that NK cells containing a single *V* allele provide better direct tumor killing in an antibody-independent manner.

Cetuximab induces anti-proliferative effects

In addition to studying cetuximab-mediated ADCC in SCCHN, we set out to evaluate the direct anti-proliferative effect of cetuximab on SCCHN, using

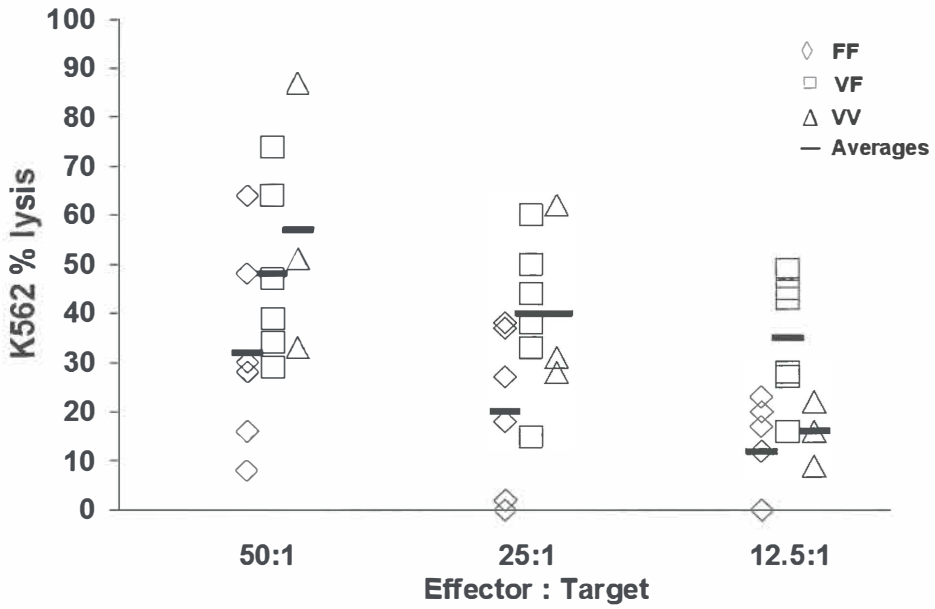

Figure 3. FcγIIIaR *V* polymorphism is associated with enhanced natural cytotoxicity against K562 tumor targets. Tumor cell line K562 was subjected to direct NK cytotoxicity in the absence of antibody. NK cells from healthy donors bearing FcγIIIaR *F/F* (open diamonds), *V/F* (open squares) and *V/V* (open triangles) alleles were tested for cytotoxicity against K562 using a standard ^{51}Cr-release assay.

thymidine proliferation assays. A significant dose-dependent inhibition on cell growth was observed on TU159 in presence of cetuximab concentrations ranging between 10pg/ml and 10µg/mL when compared to cells cultured with isotype control (Figure 4). Interestingly, there was no dose-dependent inhibition observed with TU167 and 012SCC and the effect on proliferation of these two cell lines when co-incubated with cetuximab was less sensitive compared to TU159. Thus, among the three EGFR over-expressed (>90%) cell lines, inhibition by cetuximab did not correlate with EGFR expression. In order to understand how cetuximab influences tumor growth, we employed a BrdU/7-AAD staining strategy to determine the effects of cetuximab on the cell cycle. TU159 showed an approximate 2 fold increase in the G2 phase and M phase of the cell cycle, while no overt pro-apoptotic effect was induced by cetuximab (Figure 5). Consistent with our proliferation studies, 012SCC and TU167 did not demonstrate significant reductions in DNA synthesis. Distinct from its likely role in ADCC, cetuximab may demonstrate a specific anti-proliferative effect on SCCHN. This experiment supports the ability of cetuximab in directly inhibiting SCCHN growth, in some instances, via blockade of the EGFR mechanism rather than direct cytotoxicity.

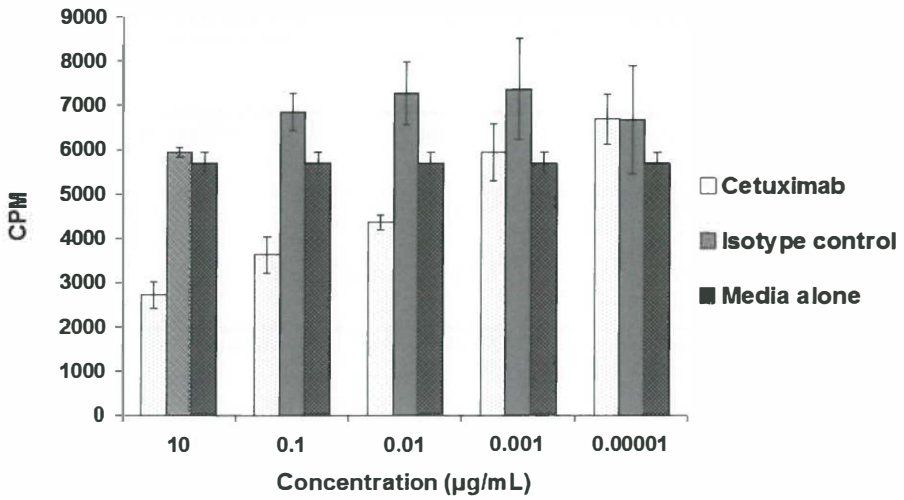

Figure 4. Dose-dependent anti-proliferative activity of cetuximab measured by [^{3}H] thymidine assays. TU159 cells were cultured in the presence of various concentrations of cetuximab for 72 h. Proliferation was measured by thymidine incorporation. Cetuximab significantly inhibited TU1 59 in a dose-dependent fashion.

Discussion

Targeted therapy with monoclonal antibodies, such as cetuximab, is an integral part of the management of a wide range of malignancies (2,8,20,25,32,35). In the present study, we demonstrate for the first time evidence for cetuximab-mediated ADCC *in vitro* among three different SCCHN cell lines. The effectiveness of ADCC is associated with FcγRIIIa donor polymorphisms recognized to enhance Fc-FcR interactions. Importantly, we also show that the presence of a *V* allele correlates with the ability of NK cells to kill sensitive targets in the absence of antibody. These data provide insight into the mechanism of action of cetuximab for SCCHN, and contribute to an increasing body of literature clarifying the role of FcγRIIIa polymorphisms in ADCC. While the clinical relevance of ADCC is difficult to establish, we provide *in vitro* data that supports its presence in SCCHN. Several recent studies also provide strong evidence supporting the impact of ADCC in targeted therapies for lymphoma, esophageal, melanoma, and colon cancer (20,25,33,35). For example, Weng and Levy have demonstrated rituximab-mediated cytotoxicity (ranging from 13.5%-100%) for lymphoma cells from 43 patients using effector cells from healthy donors (33). Recently, Kurai has provided evidence for cetuximab-mediated ADCC using low-expressing EGFR lung cancer cell lines as targets and healthy donor PMBC as effectors. In this study, Kurai demonstrated that NK cells were the effector cells responsible for ADCC and that their cytotoxicity was enhanced by IL-2 (20). Similarly, Naramura *et al.*

Table 1 Cetuximab-dependent ADCC against TU167, TU159 and 012SCC targets

cell line[a]	polymorphism		
	F/F	*V/F*	*V/V*
TU167	25% (range 2%-42%	48% (range 13%-93%	64% (range 49%-91%
sample size	n=7	n=7	n=3
TU159	33% (range 6%-66%)	40% (38% and 42%)	41%
sample size	n=4	n=2	n=1
012SCC	35% (19% and 50%)	72% (62% and 82%)	58%
sample size	n=2	n=2	n=2

[a]Data represent all ADCC experiments performed on three SCCHN cell lines with various FcγIIIa receptor polymorphism PBMC at an effector to target ratio of 50:1

demonstrated evidence for ADCC in a melanoma tumor model. These reports support our finding demonstrating that cetuximab mediates ADCC in SCCHN.

Perhaps the most compelling support for the clinical contribution of ADCC relates to the observation that polymorphic variations of NK FcγRIIIa influence clinical response and survival (32,33). This observation, coupled with the fact that NK cells are the principle effector cells facilitating ADCC, lends support to the clinical relevance of ADCC as an important mechanism of action for cetuximab. The *V* allele not only confers more efficient ADCC *in vitro,* but is also associated with better clinical outcomes. In addition to providing evidence for ADCC, our data also convincingly show that donor NK cells that contain a FcγRIIIa *V* polymorphism at position 158 demonstrate more efficient cytotoxicity against SCCHN cell lines. This important finding was seen in all three SCCHN cell lines. However, given the infrequency of homozygous *V* donors and relatively small number of samples, we did not see a statistically significant higher cytotoxicity to determine superiority of *V/V* over *V/F*. Incidentally, the frequency of the *V* allele in our sample of donors was 0.3, which is in keeping with the frequency found by others (5,33,35). These findings are significant because they may have profound implications for the identification of SCCHN patients most likely to respond to cetuximab.

Other investigators also provide support for an enhanced effect of the 158-*V* polymorphism. Hatijiharissi *et al.* demonstrated the superior effect of having a single *V* allele on ADCC against two CD20 expressing target cells lines using rituximab and NK cells from 52 healthy donors (16). Not only was rituximab-mediated ADCC activity higher with the presence of at least one *V* compared to those homozygous for *F*, but there was also a higher absolute number of CD16 receptors per NK cell for donors who expressed at least one *V* allele.

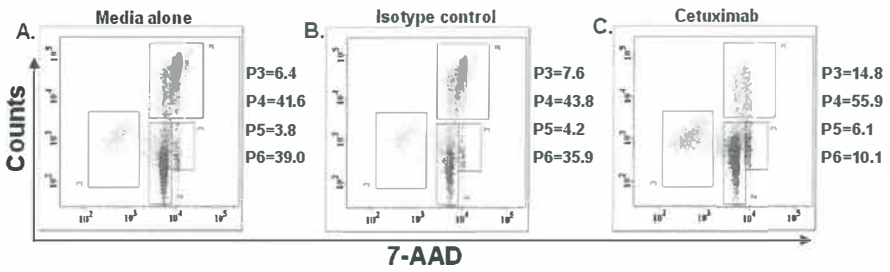

Figure 5. Cell cycling and DNA synthesis activity of TU159 in the presence of 10μg/mL cetuximab. Panels A, B and C indicate representative experiments for TU159 in media alone, 10μg/mL isotype control or 10 μg/mL cetuximab respectively. With cetuximab incubation, an increase in G2 phase and M phase (P5 quadrant) was observed compared to isotype control. S-phase (P6 quadrant) was decreased by at least 2 fold in panel C compared to panels A and B.

It also has been well established that FcγRIIIa *V* binds to human IgG1 better than the FcγRIIIa *F*, and this increased affinity of the FcγRIIIa *V* translates to an enhanced ADCC in the presence of NK cells (29,31). Optimizing the binding of FcγRIIIa to the Fc portion of targeted mAbs may offer an effective mechanism to overcome individual variability in Fc-FcR interactions. Mechanistically, these data suggest that it is not only the higher affinity binding of the Fc-FcR for the *V* allele that confers ADCC benefits, but the higher absolute number of CD16 receptors among individuals with the *V* allele may contribute to the superiority observed by many investigators.

Additionally, there have been clinical studies reporting that individuals homozygous for the *V* allele have an advantage over those that are either heterozygous *V/F* or homozygous *F*. A retrospective clinical review of 136 lymphoma patients demonstrated a clinical 5-year progression free survival (PFS) for *V/V*, *V/F* and *F/F* equaling 77%, 38%, and 48%, respectively (33). Another analysis of 87 lymphoma patients by Weng *et al.* confirmed the clinical benefits of homozygous *V* with a 2-year progression-free survival (PFS) for *V/V*, *V/F* and *F/F* that was 45%, 12%, and 16%, respectively (32). In contrast to the *in vitro* studies, these clinical findings suggest that lymphoma patients with the homozygous *V* allele may experience better clinical outcomes when treated with rituximab. While there is compelling support for the positive effect of a *V* allele on ADCC, there are studies that do not maintain this position. Zhang *et al.* recently suggested that *V/V* at amino acid position 158 was unfavorable among 35 patients with disseminated colon cancer (35). However, only 5 (14%) of the patients in this study were *V/V* and the benefit attributed to having an *F* was not statistically significant in this

small sample. No direct measure of ADCC was performed in this study to correlate with their observed clinical outcomes. Additionally, because many end-stage patients are relatively immunosuppressed and have significantly impaired NK function (20), the findings in this small, single institution study should be viewed in context. Other small studies were not able to provide evidence for or against the role of polymorphisms in outcomes using targeted antibody treatments, as well (13,22). While additional studies are necessary, our findings demonstrating the benefit of a *V* allele for cetuximab-mediated ADCC are supported by larger, reproducible clinical and *in vitro* ADCC studies described previously.

One of the principle goals of this study was to evaluate cetuximab-medicated ADCC in SCCHN, however we acknowledge that there are additional proposed mechanisms for cetuximab's anti-tumor effect, such as complement dependent cytotoxicity (CDC), competitive inhibition, or possibly direct pro-apoptotic effects (21,28). Crystallography studies show that cetuximab interacts with the ligand binding domain of the receptor, resulting in steric hindrance of ligand binding and subsequent dimerization of the molecule, which prevents EGFR autophosphorylation and activation (17). Therefore, we also evaluated the direct inhibition of the EGF receptor via proliferation and cell cycle experiments.

Our proliferation studies demonstrate significant inhibition only against TU159 in a dose dependent fashion, resulting in cell cycle arrest at the S-phase, with an increased shift at the G_0/G_1 phase. We did not observe significant apoptosis in any of the three cell lines. Thus, it appears that this anti-proliferative property of cetuximab relates to direct inhibition of ligand binding. However, we did not observe significant levels of cetuximab inhibition with TU167 and 012SCC compared to TU159, despite similar EGFR expression among the three cell lines. Our findings demonstrate that inhibition did not correlate with EGFR expression. In fact, some EGFR over-expressed cancers demonstrate an ability to become less sensitive to cetuximab and proliferate in a manner that is less dependent on the EGFR/tyrosine kinase pathway (17,27,28,34). Interestingly, even though there is variation in cetuximab-mediated inhibition among the three tumor targets, all three cell lines show a strong susceptibility to cetuximab-mediated ADCC.

In addition to ADCC, NK cells possess other important cytotoxic functions. Some of these include antibody-independent mechanisms of cytotoxicity, and their effectiveness in this capacity may also be associated with FcγRIIIa polymorphisms. Interestingly, we observed an unexpected, not previously described finding that

donor NK cells with a *V* allele were associated with superior cytotoxicity against K562 in an antibody-independent mechanism. This is the first report documenting an association with the FcγRIIIa polymorphisms in antibody-independent cytotoxicity with NK. Thus, in addition to demonstrating more efficient ADCC against SCCHN cells, NK cells with a *V* allele were also associated with a statistically significant higher cytotoxicity against NK susceptible K562 in the absence of antibody (Figure 6). The mechanism for the enhanced cytotoxic potential of NK cells bearing a *V* allele against NK sensitive targets is unknown. Sivori describes NK cells with phenotypically more proficient cytotoxic activity that have a higher surface density of NKp46, an important NK natural cytotoxicity receptor (NCR) (30). Others have confirmed that individual donors have populations of NK with heterogeneous surface densities of NCR as well as other NK co-receptors that correlate with NK cytotoxic activity (3,23,24,30). Additional studies are necessary to determine if the same polymorphisms that are associated with enhanced ADCC in SCCHN may also correlate with a phenotype that confers superior cytotoxicity.

The intent of our study was to determine if FcγRIIIa polymorphisms are important in predicting response to cetuximab in SCCHN tumor cells. We demonstrate, for the first time, that cetuximab can induce NK mediated ADCC in SCCHN *in vitro*. Furthermore, we show that CD16 polymorphisms play a critical role in the efficiency of ADCC in SCCHN. Specifically the addition of a *V* allele provides enhanced ADCC. This observation may prove to be an important marker for identifying individuals who can derive the greatest benefit from cetuximab-based therapies. An additional finding is that the *V* allele may not only be associated with enhanced ADCC, but may also correlate with an enhanced antibody-independent cytotoxicity. Our observations provide insight into the mechanism by which cetuximab may mediate therapeutic effects *in vivo* and provide a rationale for developing clinical trials to determine the role of FcγRIII polymorphisms and response to cetuximab in patients with SCCHN.

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PART C

Scientific possibility and clinical reality: Lessons learned and hurdles to overcome

7

Trojan vaccines induce MAGE-A3 and HPV-16 immunity in patients with head and neck carcinoma

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Abstract

We performed a pilot study using Trojan vaccines in patients with advanced squamous cell carcinoma of the head and neck (SCCHN). These vaccines are composed of HLA class I and HLA class II restricted MAGE-A3 or HPV-16 derived peptides, joined by furin-cleavable linkers, and linked to a "penetrin" peptide sequence derived from HIV-TAT. Thirty-one SCCHN patients were screened for the trial and five were enrolled. Enrolled patients were treated with 300μg of Trojan peptide supplemented with Montanide and GM-CSF at 4-week intervals for up to four injections. Following vaccination, PBMC from 4/5 patients recognized both the full Trojan constructs and constituent HLA class II peptides, while responses to HLA class I restricted peptides were less pronounced. These data suggest that this treatment regimen appears to have acceptable toxicity and elicits measurable systemic immune responses against HLA class II restricted epitopes in a subset of patients with advanced SCCHN.

Introduction

The therapeutic options for patients with advanced squamous cell carcinoma of the head and neck (SCCHN), who fail surgical extirpation and/or treatment with chemoradiotherapy, are generally limited to observation or palliative chemotherapy (1). In order to improve the outcomes for this patient population, investigators have explored the use of immunotherapy in the form of peptide vaccines in an attempt to stimulate therapeutic antitumor immune effector responses. Traditional peptide cancer vaccines have focused on priming HLA class I (HLA-I) restricted responses, adhering to the premise that efficient stimulation of antigen-specific CD8 T cells, is both necessary and sufficient for effective antitumor immunity (2). However, the limited clinical efficacy of HLA-I restricted vaccines in isolation (3), has increased attention on the potential utility of simultaneously stimulating HLA class II (HLA-II) mediated CD4 responses, which may be necessary for the development and/or maintenance of antitumor immunity (4, 5). A first step to capitalize on this strategy is to develop vaccines against well characterized antigens which employ both HLA-I and II peptide-specific sequences.

In order to both generate HLA-I and II restricted T cell responses in humans and overcome historic problems associated with extracellular proteolysis of short HLA-I peptides, we employed a "Trojan" peptide approach. Trojan peptide-based vaccines contain a "penetrin" peptide sequence derived from HIV-TAT (RKKRRQRRR) which allows the entire peptide to translocate through the cell membrane and penetrate directly into the Endoplasmic Reticulum (ER) and Golgi apparatus (Golgi) where they can form peptide-HLA complexes (6). In addition, this strategy utilizes furin-cleavable linkers to join multiple HLA-I and II peptide-epitopes, enabling release of the individual peptide-epitopes in the Golgi, where furin endopeptidase resides (7). These Trojan peptide-based vaccines appear to be relatively resistant to proteolysis and do not require proteosomal processing and transport by TAP, since they penetrate directly to the ER and Golgi (6). Perhaps more importantly, these functional attributes are associated with improved outcomes in both prophylactic and therapeutic mouse tumor models (7).

For purposes of this study we designed two separate Trojan constructs, using previously described HLA-I and II T cell epitopes of the human papilloma virus type 16 (HPV-16) and MAGE-A3 tumor associated antigens. MAGE is a cancer testis antigen originally found in melanomas but later identified in other epithelial cell malignancies (8), while HPV-16 is a papillomavirus which is associated with SCCHN of the oro- and hypopharynx (9, 10). The choice of HPV-16 was premised

on several considerations including its high prevalence in SCCHN, tumor specificity and the presence of well-defined peptide epitopes (9-13). Similarly, MAGE-A3 was selected based on its high levels of expression in SCCHN and the availability of well characterized HLA-I and II immunostimulatory epitopes (14-16). Prior to injection, the Trojan peptides were solubilized in Montanide ISA 51 and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF, Sargramostim), as a means to promote DC migration to the site of vaccination and to enhance antigen presentation (17). In the first group of patients both an HLA-A*02 positive genotype and expression of HLA-A*02 on the tumor cell surface were mandated for study entry. This original study design was premised on the fact that the HLA-I peptides in the vaccine are HLA-A*02 specific and could only target tumors expressing this HLA-I allele (18). However, based on our interval findings that the peptides predominantly stimulated responses against the HLA-II epitopes, whose allele restrictions are recognized to be promiscuous, the inclusion criteria were modified to include all patients with either MAGE-A3 or HPV-16 positive tumors.

Thirty-one patients with recurrent or metastatic SCCHN were screened for this pilot study and five were enrolled. Three patients received the MAGE-A3 and two patients received the HPV-16 vaccine. Although one patient with a known stable brain metastases experienced a serious adverse event (SAE), the Trojan vaccines were well tolerated and toxicities in general did not require or prolong hospitalization. In four out of five immunized patients, durable IFNγ responses were observed in response to both the intact Trojan peptide and the HLA -II specific peptide. In addition, Trojan peptide-specific IgG was detected in plasma from two patients. By Elispot, Trojan peptide-specific HLA-I IFNγ-producing cells were identified in the peripheral blood of one of the MAGE-A3 immunized patients. To the best of our knowledge, this study is the first to report the use of Trojan peptide vaccines in humans and suggests that this strategy might provide a useful platform for priming antigen specific T cell responses in patients with head and neck carcinoma.

Materials and methods

Patient population, study protocol and treatment schedule

Between December 2005 and March 2007, 31 patients were screened for their eligibility to enroll in a phase I clinical study designed to evaluate toxicity and immunogenicity of two Trojan (HPV-16 and MAGE-A3) peptide-based vaccines. All experimental work related to human subjects in this study was approved by the

University of Maryland's Institutional Review Board. Written informed consent was provided according to the Declaration of Helsinki. Enrollment criteria included (i) patients with recurrent or metastatic SCCHN not amenable to treatment with standard therapy or patients who refused standard treatment, (ii) the presence of the HLA-A*02 genotype and (iii) either MAGE-A3 or HPV-16 expression in the tumor. Importantly, based on interim data analysis demonstrating that the immune responses were predominantly HLA-II restricted, we eliminated the HLA-A*02 restriction in the latter part of the study.

The vaccine dose used in the trial was 300μg of Trojan peptide, with 1.2 milliliters (mL) of Montanide ISA 51 (Seppic, France) and 100μg/m^2 of GM-CSF) (Berlex, Seattle, WA). This vaccine dose was chosen because it is well within the range of previous peptide vaccine trials for cancer and it was well tolerated by the majority of patients while providing evidence of immunological efficacy (2). Peptides were reconstituted with 0.5 mL of sterile water for injection and each vial was mixed with Montanide ISA 51 and GM-CSF in an empty vial. The total contents of the mixture were drawn up in a syringe and pushed back into the vial. This mixing step was repeated 25 times to make unified emulsions, and final emulsions were drawn up in multiple syringes for injection. All vaccinated patients were injected subcutaneously in the inguinal region with the appropriate Trojan peptide vaccine at 4-week intervals for up to four injections as indicated in figure 1. The primary indications for giving fewer than four injections were drug-related toxicity (patient 1) or disease progression (patients 2 and 4). After the final injection, patients returned for follow-up visits every three months.

Trojan-peptide based vaccines and individual peptides

The MAGE-A3 Trojan peptide and the HPV-16 Trojan peptide were manufactured by Multiple Peptide Systems (San Diego, CA) and processed and vialed by Merck Biosciences AG (Laufelfingen, Switzerland). Patients were immunized with either the MAGE-A3- or the HPV-16-specific Trojan peptide vaccine. Specific sequences of the Trojan vaccines and individual antigens to determine vaccine-induced immune responses are listed in table 1. The peptides were studied under IND # 10927 (NCI www.clinicaltrials.gov # NCT00257738).

Isolation of PBMC and tissue/tumor infiltrating lymphocytes

Whole blood samples were collected prior to the initiation of treatment, within 24 hours before each vaccination and then at defined follow-up intervals.

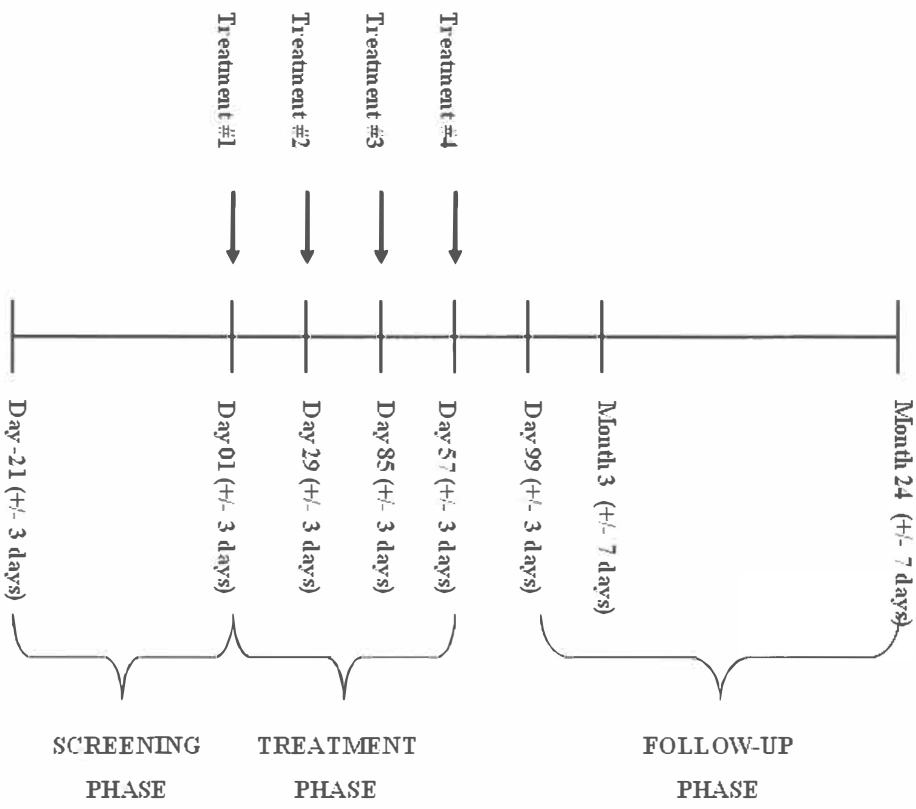

Figure 1. Immunization schedule. All enrolled patients were immunized subcutaneously in the inguinal region at 4-week intervals for up to four injections. After the final immunization, patients returned for follow-up visits every three months.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. Tissue/tumor infiltrating lymphocytes (TIL) were isolated as described by Whiteside et al. (19), cryopreserved in RPMI-1640 supplemented with 10% normal human AB serum (NHS) and 10% DMSO using an automated cell freezer (Gordinier Electronics, Roseville, MI) and stored in the vapor phase of liquid nitrogen for later use. If possible, PBMC/TIL isolated from separate procurement dates were simultaneously thawed and assayed to assess *in vitro* function. All experiments were performed in RPMI-1640 supplemented with 10% NHS, 2% HEPES, 1% P/S and 1% L-Glutamate (hereafter indicated as cRPMI).

HLA typing

DNA was extracted from a portion of the PBMC using a QIAamp DNA mini kit (Qiagen) and the alleles of genes encoding HLA-ABDR were determined by the

Micro SSP™ HLA-ABDR DNA typing kit (One Lambda, Canoga Park, CA) as per manufacturer's instructions. All HLA-A*02 positive patients were further analyzed for the genes encoding HLA-A*02 by the Micro SSP™ HLA-A*02 DNA typing kit (One Lambda) as per the manufacturer's instructions.

MAGE-A3 and HPV-16 PCR

Total RNA was extracted from squamous cell tumor biopsies using a Qiagen RNeasy Mini kit as per manufacturer's instructions followed by cDNA synthesis using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The melanoma antigen gene family (MAGE) consists of twelve highly homologous genes encoded on human chromosome Xq28 (20). *MAGE-A3* (NM_005362) is highly homologous to *MAGE-A1,2,4,5,6* and *12* in the region of the Trojan peptide. We observed that amplification and sequencing of MAGE-A3 from tumor samples using previously published primers for MAGE-3, was not specific for MAGE-A3 (15). Therefore, new PCR primers were designed. For specific amplification of *MAGE-A3* from cDNA primers MA3_F4 AAC GAG CGA CGG CCT GAC G and MA3_R4 GTG GAA ACT AAG GGA TGC were designed, which yield a PCR product of 1221 bp. For specific amplification of *MAGE-A3* from genomic DNA primers MA3_F1 GTG GAA ACT AAG GGA TGC and MA3_R4 were designed, which yield a PCR product of 736 bp. Primer MA3_R4 is specific for *MAGE-A3* and forms a 3' C:C mismatch with the sequences of *MAGE-A2,4,5,6* and *12*, which is predicted to decrease PCR efficiency by greater than 99% (21). To confirm the specificity of the amplified products, total RNA and genomic DNA were extracted from three different SCCHN cell lines, TU159, 012SCC and HW12, which express multiple MAGE proteins. An *Eco*RI restriction enzyme recognition site is present in nucleic acid 1017 of *MAGE-A3* (NM_005362) but not in the potentially amplified sequence of any of the other MAGE genes. The specificity of the amplification was tested by restriction digestion of the PCR products with an *Eco*RI restriction enzyme followed by direct sequencing of PCR products (supplemental figure 1A). Complete digestion of the PCR product indicated specific amplification in all samples tested, compared with non-specific amplification with previously published MAGE-3 primers (supplemental figure 1B). Amplification of HPV16 cDNA was performed using primers E7_F GCT CAG AGG AGG AGG ATG AA and E7_R GCC CAT TAA CAG GTC TTC CA to amplify HPV16 E7 (255 bp product) (10). PCR products were then verified using direct sequencing (supplemental figure 1C). All PCRs were performed using ABGENE 2X ThermoPrime ReddyMix (Thermo Fischer Scientific, Rockford, IL) with 1.5mM MgCl2 and a PCR protocol that consisted of ten cycles that began with an

annealing temperature of 63 °C and decreased by 0.5 °C per cycle followed by 32 cycles with an annealing temperature of 58 °C and a one minute elongation time.

Immunohistochemistry

For HLA-A*02 immunohistochemical detection, frozen sections were cut at five micron thickness from the patient and control tissues and mounted on charged slides. The slides were incubated in cold acetone for ten minutes prior to processing in a DAKO Autostainer with an anti-HLA-A*02 antibody (SB03-111, kindly provided by Dr. S. Ferrone, University of Pittsburgh, PA, USA and AEC substrate as per manufacturer's instruction. Staining with lymphocyte markers CD3, CD8, and CD68 (all from Ventana, Tucson, Arizona) and CD4 (Biocare, Concord, CA) was performed on formalin fixed paraffin-embedded tissue. Deparaffinization, rehydration and epitope retrieval were performed following standard procedures. Staining was performed on a Ventana Benchmark-XT stainer using an AP detection system.

Trojan-specific T cell response monitoring

IFNγ Recall Elispot assay

96-well nitrocellulose-bottom (Elispot) plates (Millipore, Molsheim, France) were pre-coated with IFNγ at a final concentration of 15μg/ml (Mabtech, Nacka, Sweden). Plates were then washed six times with sterile PBS and blocked with 100μl/well of RPMI 1640 supplemented with 10% NHS. After 60 minutes of incubation, the blocking media was discarded and 2 x 10^5 PBMC were plated in triplicate wells. Next, Trojan and individual peptides were added to appropriate wells at a final concentration of 10μg/ml and plates were incubated at 37 °C, 5% CO_2. After 48 hours of incubation, cell supernatants were discarded and Elispot plates developed using a Mabtech development kit. In brief, plates were washed six times with 1X PBS and subsequently incubated for two hours at room temperature with 100μl/well of biotinylated anti-human IFNγ (7-B6-1, Mabtech) antibody (mAb) at a final concentration of 1μg/ml. After two hours, plates were washed six times with 1X PBS and incubated for one hour with 100μl/well of Streptavidin-HRP (Mabtech) at room temperature. Finally, Elispot plates were washed six times with 1X PBS and developed for 30 minutes with 100μl/well of peroxidase substrate AEC kit (Vector Laboratories, Burlingame, CA), followed by rinsing under running tap water. Plates were stored over night in the dark at room temperature, and spots were counted using a VersaScan microplate reader (Velocity 11, Palo Alto, CA).

Table 1 Trojan-vaccines and individual peptides

antigen	amino acid sequence[a,b,c]	HLA-restriction
MAGE-Trojan	KVAELVHFL/**RVKR**/FLWGPRALV/**RVKR**/VIFSKASSSLQL/*RKKRRQRR*	A2, DR4/DR7
MAGE-CTL_1	KVAELVHFL	A2
MAGE-CTL_2	FLWGPRALV	A2
MAGE-HTL	VIFSKASSSLQL	DR4/DR7
HPV-Trojan	TLGIVZPI/**RVKR**/PAGQAEPDRAHYNIVTFZZKZD/*RKKRRQRRR*	A2, DR1/DR4/DR13/DR15
HPV-CTL	TLGIVZPI	A2
HPV-HTL	PAGQAEPDRAHYNIVTFZZKZD	DR1/DR4/DR13/DR15
control	YIGEVLVSV	HA-2[d]

[a]RVKR; furin-cleavable linkers are indicated in Bold
[b]RKKRRQRRR; HIV-TAT translocating domain is indicated in Italic
[c]Z = cyclophenyl alanine
[d]Human minor histocompatability antigen HA-2

To assess pre-coating efficiency, cells were stimulated with Concanavalin A (CON-A, 1μg/ml) and wells with cells alone and control peptide served as background control and negative controls, respectively. The mean total of IFNγ spot-forming cells (SFC) in triplicate wells were determined based on the difference between the frequency of spots obtained with the vaccine-specific peptides and the control peptide and is expressed as number of SFC as indicated in the figure legends. Specific sequences of the Trojan vaccines, individual vaccine-specific antigens and control antigen to determine vaccine-induced immune responses are listed in table 1.

IFNγ re-stimulation Elispot assay

To amplify T cell responses, PBMC were re-suspended at 2 x 10^6 cells/ml in cRPMI supplemented with GM-CSF (50 ng/ml) and IL-4 (25 ng/ml). Next, PBMC were plated at 2 x 10^6 per well in 24-well plates and half of the wells were re-stimulated with the appropriate Trojan vaccine (10 μl/ml) and incubated at 37° C, 5% CO_2. After 48 hours of incubation, cells were harvested, re-suspended at 1 x 10^6 cells/ml in cRPMI supplemented with IL-7 and IL-15 (both at 5 ng/ml) and plated at a final concentration of 10^6 cells/well in a 24-well plate and incubated at 37 °C, 5% CO_2. After seven days, cells were harvested, washed and plated at 1 x 10^5 cells/well in triplicate wells in 96-well IFNγ pre-coated Elispot plates. Trojan-vaccine and control peptides were added to the appropriate wells (10μg/ml) and the plates cultured for 24 hours at 37° C, 5% CO_2. After 24 hours of incubation, Elispot plates were developed as described above.

MAGE-Trojan and HPV-Trojan IgG ELISA

Plasma samples from the vaccinated patients were assayed for antibodies against either MAGE- or HPV-Trojan peptides by ELISA. In brief, ELISA plates (NUNC 445101, Raskilde, Denmark) were coated overnight at 4 ºC with 1 μg MAGE- or HPV-Trojan vaccine per well. Negative control wells included no vaccine, vaccine alone, secondary antibody alone and vaccine plus secondary antibody. After overnight incubation and subsequent washing, plasma samples were diluted in blocking buffer (as indicated in the figure legend) and 100μl of diluted plasma was added to triplicate wells. After one hour of incubation at room temperature, plates were washed five times with PBS supplemented with 0.05% Tween 20 (1X PBST) and incubated for another hour with 100 μl of peroxidase-conjugated goat anti-human IgG (dilution 1:1000, Kirkegaard & Perry, Gaithersburg, MD). Finally, 100 μl of 3,3', 5,5'-Tetramethylbenzidine (TMB, Kirkegaard & Perry, Gaithersburg, MD) substrate was added and plates were incubated for up to 30 minutes at room temperature. OD values were monitored at 650 nm to not exceed 1.3. Reactions were stopped with 100 μl 1N HCl and OD values determined at 450nm. Baseline levels of samples tested with no vaccine were subtracted from those with vaccine. OD values at 450nm were graphed and reported.

Tetramer Analysis

PE-labeled tetramers were assembled by Beckman Coulter (Fullerton, CA). Tetramers specific for MAGE-A3 CTL_1 (KVAELVHFL), MAGE-A3 CTL2 (FLWGPRALV), HPV-16 CTL (TLGIVZPI) and control HLA-A*02 restricted tetramer (YIGEVLVSV) were used. For tetramer staining, 20μl aliquots of tetramer were directly added to 10^6 PBMC. Cells were incubated for 30 minutes at room temperature in the dark. Next, 5μl aliquots of CD3 and CD8 mAb were added for surface staining, and the cells were incubated for an additional 30 minutes at 4 ºC in the dark. After incubation, cells were washed twice with FACS buffer, resuspended in 500μl of FACS buffer and analyzed within two hours of staining. A minimum of 100,000 events were acquired using a BD LSR II flow cytometer and analyzed with BD FACS DIVA Software (BD Biosciences).

Statistics

The number of IFNγ producing PBMC observed in separate Elispot wells were treated as events that occurred in a pseudo-time period. Poisson regression was therefore used to test if there were any significant differences in the mean events between pre-vaccine and post-vaccine time-points for various vaccine and epitope treatments in each patient. The analysis was implemented with the SAS 9.2 (SAS

Institute Inc., Cary, NC) GENMOD procedure with Poisson distribution. Over-dispersion (variance > mean) of the model was adjusted by using 'dscale' option in the model statement. The significance level was set at 5%. P <0.05 was considered to be significant.

Results

Patient characteristics, HLA results and MAGE-A3/HPV-16 expression

A total of 31 patients were screened for enrollment in this pilot study and the demographic data on all screened study patients is provided in table 2. All screened patients had histologically proven SCCHN and had either failed therapy or refused standard therapy. HLA typing was performed on all potential study patients, followed by a tumor biopsy to determine MAGE-A3, HPV-16 and HLA-A*02 expression within the tumor. Among the 31 patients who were HLA typed, 11 patients were determined to be HLA-A*02 positive. Nine of the HLA-A*02 positive patients and two of the HLA-A*02 negative patients were subjected to a tumor biopsy. Two HLA-A*02 positive patients were found to be negative for HLA-A*02 at the tumor level (supplemental figure 2). These findings are consistent with previous studies from others and us showing that loss of HLA-I expression is a relevant mechanism of immune escape in SCCHN (22). Of the 11 patients biopsied, four were shown to express MAGE-A3 and two were proven to express HPV-16. One of these patients expressed both antigens. In contrast, four patients expressed neither of the required antigens. In summary, five SCCHN patients were enrolled (including one "single use exemption" secondary to a diagnosis of myelodysplastic syndrome) in this pilot study and treated with up to four immunizations with one of the Trojan vaccines (table 3).

Clinical Outcomes and Toxicities

None of the five patients immunized demonstrated a complete response (CR) or partial response (PR) by RECIST criteria. Patient 5 is alive at 31 months after trial entry. Patient 1 lived for 27 months while patients 3, 4 and 2 died at 24, 8 and 4 months, respectively. Patient 1 received only one vaccine and was removed from study due to a serious adverse event. A follow up CT scan five months following vaccine administration showed stable disease. Patient 2 was removed from study following two vaccinations due to disease progression. Patients 3 and 4 showed disease progression by CT scan two months following trial entry. Patient 5 showed stable disease following two vaccinations and underwent tumor resection three months following the final vaccination.

Table 2 Demographics of screened study patients

number of patients	31	
male	25	
female	6	
median age (years); Range (years)	56 (39-79)	
stage	IV	
primary site: oral	6	
oropharynx	11	
hypopharynx	4	
larynx	5	
nasal/paranasal cavities	2	
nasopharynx	1	
unknown	1	
HLA phenotype: HLA-A*02$^+$ PBMC	11/31	(35%)
afro-American	2/10	(20%)
Caucasian	9/21	(38%)
HLA-A2$^+$ tumor	7/9	(78%)
Tumor-antigen expression[a]: MAGE-A3$^+$	4/11	(36%)
HPV-16$^+$	2/11	(18%)
MAGE-A3$^+$/HPV-16$^+$	1/11	(9%)

[a] including two tumor biopsies of HLA-A*02 negative patients

Importantly, our survival data must be taken in context with the fact that these patients received other surgical and/or chemotherapeutic regimens after vaccination.

NIH Common Toxicity Criteria were employed to determine the clinical toxicities associated with the Trojan vaccines. Toxicities observed after the first immunization and worst toxicity observed after any immunization are summarized in table 4 and 5, respectively. One patient experienced a serious adverse event (SAE) which is particularly noteworthy. Specifically, this patient with a known stable brain metastases (figure 2A) developed right sided hemiplegia, right-sided facial weakness and speech impairment, 24 days after her first vaccination. T2-weighted MRI revealed marked progression of increased signal surrounding the cerebral metastasis consistent with increased cerebral edema (figure 2B). In addition, there was a rim of decreased signal that developed along the periphery of the metastasis, which although nonspecific, is an imaging feature that could be consistent with the development of surrounding cellular/lymphocytic infiltration. With steroid treatment, these findings subsided and her neurological symptoms resolved (figure 2C).

Table 3 Demographics and HLA status of immunized patients

patient	age (years)	sex	AJCC	HLA ABDR[a]	HLA A2 (tumor)	Trojan HTL IFNγ	Trojan IgG
1	50	F	IV	A2, A24, B77, B44, DR4,	60%-80%	+	-
2	65	M	IV	A2, A66, B7, B71, DR15,	60%-80%	-	-
3	70	M	IV	A2, A-, B7, B13, DR15, DR-	40%-60%	+	+
4[b]	47	M	IV	A3, A33, B-, B35, DR1,	n/a	+	-
5	55	M	IV	A2, A3, B7, B35, DR12,	80%-100%	+	+

[a]Trojan-vaccine specific HLA-I and II restrictions are underlined

[b]single use exemption, due to a secondary diagnosis of myelodysplastic syndrome.
n/a, not applicable

In vitro Elispot interrogation of PBMC, demonstrated strong recognition of both the Trojan and HLA-II restricted peptides post-immunization (figure 2D). We postulate that this cerebral edema is the result of a vaccine-induced inflammatory reaction, although direct biopsy evidence is lacking.

Trojan peptide-based vaccines induce robust and long lasting systemic vaccine-specific HLA-II T cell responses

To evaluate the vaccine-specific T cells responses, PBMC obtained from patients at baseline, within 24 hours prior to each vaccination and at completion or discontinuation of study were evaluated both directly *ex vivo* and after *in vitro* sensitization using IFNγ Elispot. In the absence of prior *in vitro* exposure (hereafter called "recall Elispot") Trojan-vaccine specific post immunization T cell responses were observed in PBMC from patient 3 ($P=0.0045$, $P=0.0100$ and $P<0.0001$ after vaccination number 2, 3 and 4, respectively), patient 4 ($P=0.0016$ after vaccination number 3) and 5 ($P<0.0001$, $P=0.0270$, $P=0.0001$, $P=0.0004$ and $P=0.0007$ after vaccination number 1, 2, 3, 4 and after 3 months of follow-up, respectively). Furthermore, transient Trojan-specific HLA class II responses were observed in PBMC from patient 3 after vaccination number 2 ($P=0.0021$) and after vaccination number 3 in PBMC from patient 4 ($P=0.0041$). Examples of MAGE-A3 (patient 3) and HPV-16 (patient 5) recall assays are shown in figure 3A and B, respectively.

Trojan-specific immunity was significantly enhanced when PBMC were subjected to a single *in vitro* exposure with the respective Trojan-vaccine. Specifically, MAGE- or HPV-Trojan specific immunity was detected in four out of five immunized patients ($P<0.0001$ in all four patients at any given time-point) after one single *in vitro* re-stimulation. Examples of MAGE-A3 (patient 3) and HPV-16 (patient 5) *in vitro* sensitization assays are shown in figures 3C and D, respectively.

Table 4 Toxicity after the first immunization

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Diarrhea	1			
Nausea		1		
Vomiting	1	1		
Head/Neck edema		1		
Chills		1		
Dehydration		1		
Infection		1		
BUN[a]				
Pain - musculoskeletal		1		
Pain – injection site		1		
Soft-tissue-joint-function		1		
Hemorrhage/Bleeding	1			
Cardiac-ischemia infarction		1		
Hemoglobin - low		1		
Total	3	11	0	0

[a] BUN, blood urea nitrogen

Table 5 Worst toxicity observed during any immunization

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Dyspnea		1	1	
Nausea			1	1
Vomiting		2		
Fatigue		1		
Head/Neck edema				1
Chills		1		
Neuropathy – motor				1
Neuropathy – Cranial nerve			1	
Infection			1	
Pain – musculoskeletal		1	2	
Pain – injection site		1		
Soft-tissue-joint-function		1		
Hemorrhage/Bleeding		1		
Cardiac-ischemia infarction		1		
Hemoglobin - low				1
Diplopia			1	
Upper respiratory - fistula	1			
Total	1	10	7	4

Importantly, *in vivo* priming and additional *ex vivo* amplification also resulted in robust Trojan vaccine-induced responses to the HLA-II peptide in these four patients (figure 3C and 3D). These responses appeared to peak after the second or third vaccination with $P<0.0001$ at any given time-point. Quantitative levels of immune responses observed at baseline, after the second immunization and after the fourth immunization in both patients who completed the full vaccination schedule are summarized in supplemental figure 3. To exclude non-specific immune responses, in two MAGE-A3 vaccinated patients (patients 1 and 2) the HPV-Trojan vaccines were added to the recall Elispot assay as an additional internal control. In these two assays, nonspecific responses were absent (data not shown). In addition, the HIV-TAT sequence was tested in a re-stimulation HPV-16 Elispot assay (patient 4), and no significant differences in SFC were observed between wells containing the HIV-TAT or negative control peptides (data not shown). In contrast, by recall Elispot, no systemic responses could be detected to the HLA-I epitopes present in the Trojan vaccines.

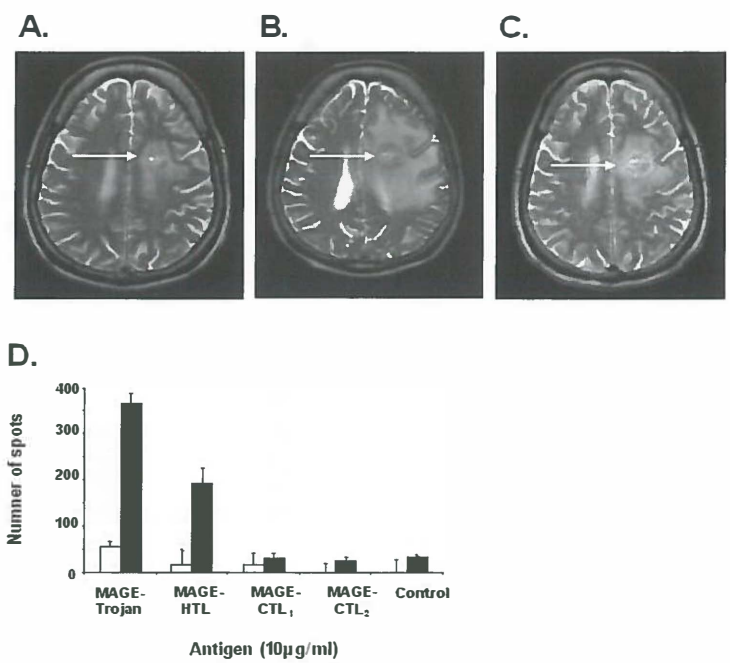

Figure 2. T2 weighted brain MRI images of patient 1 and Trojan vaccine-specific immunity. Left **(A)** image obtained eight weeks prior to vaccination (1/30/06), middle **(B)** image obtained during neurological event (4/21/06), and right **(C)** image obtained following resolution of neurological symptoms (7/14/06). Arrows indicate the site of the cerebral metastasis. PBMC were re-stimulated once with MAGE-Trojan vaccine and cultured in the presence of IL-7 (5 ng/ml) and IL-15 (5 ng/ml). After seven days, cells were evaluated for MAGE Trojan-specific HLA-I and II peptide **(D)** specificity by IFNγ Elispot assay. Number of spots per 100,000 PBMC is shown.

One transient Trojan-vaccine specific immune response was detected after *in vitro* re-stimulation in PBMC from patient 3 with P=0.0210 (MAGE-CTL_1) and P=0.0430 (MAGE-CTL_2) after receiving three immunizations. In general, the lack of response was not secondary to antigen specific T cell anergy, as tetramer analysis revealed no robust and long-lasting increase in the number of antigen-specific CD8 T cells over time (supplemental figure 4). These data demonstrate that the Trojan peptide vaccines, when administered in combination with Montanide ISA 51 adjuvant and GM-CSF, induce systemic immunity to both the entire Trojan construct and the HLA-II restricted epitopes. Furthermore, the fact that the responses to the intact Trojan constructs were higher than the responses to their constituent epitopes, suggests that natural cleavage of the Trojan peptides creates novel HLA-II restricted epitopes.

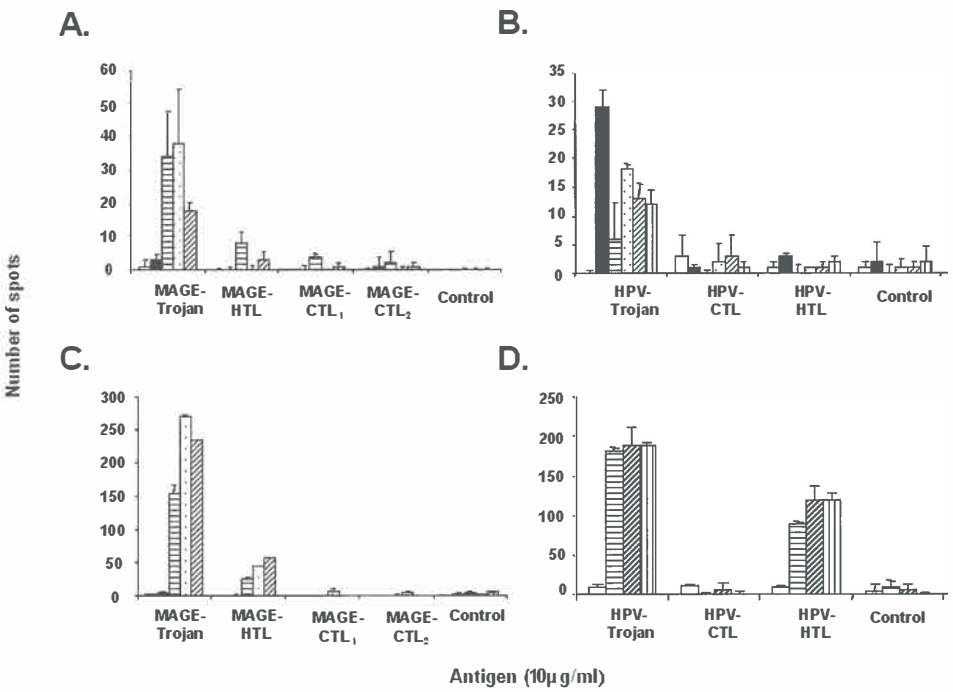

Figure 3. Trojan peptide-based vaccines induce Trojan-specific precursors *in vivo*. PBMC collected before immunization and at indicated time-points after immunization were evaluated for Trojan specificity by IFNγ Elispot assay. Direct *ex vivo* measurement of T cell reactivity against MAGE-Trojan **(A)** HPV-Trojan **(B)** and individual Trojan-specific HLA-I and II peptides **(A+B)**. Number of spots per 200,000 PBMC is shown. Error bars represent SD from triplicate wells. Control values are shown without subtraction from experimental values. PBMC were re-stimulated once with MAGE-Trojan or HPV-Trojan vaccine and cultured in the presence of IL-7 (5 ng/ml) and IL-15 (5 ng/ml). After seven days, cells were evaluated for MAGE-Trojan **(C)**, HPV-Trojan **(D)** and individual Trojan-specific HLA-I and II peptide **(C+D)** specificity by IFNγ Elispot assay. Number of spots per 100,000 PBMC is shown. Error bars represent SD from triplicate wells. * P<0.05

Alternatively, it is possible that the observed responses were biased by our methodology, where we initially stimulated with the Trojan construct and used the HLA-I or II restricted peptides for re-stimulation. Further studies will be required to clarify this issue.

Trojan peptide-based vaccines induce antigen specific IgG responses

It is well established that, under most conditions antibody class switch recombination (CSR) requires T cell help. Furthermore, regulatory T cells are recognized to inhibit antigen specific CSR by both direct suppression of B cell function and indirect inhibition of the T helper response (23). Therefore, in order to understand the functional activity of the CD4 T cell responses, we interrogated the IgG titers to the Trojan peptide vaccines. Vaccine-induced immune responses were demonstrated in patient 3 (MAGE-A3 Trojan-specific IgG) and patient 5 (HPV-16 Trojan-specific IgG) (figure 4A and 4C, respectively). These responses were durable up to follow-up month 3 and, in one case, were observed even at a 1/5000 dilution. Furthermore, IgG subtyping of the patient with the highest titers (patient 5) revealed that these responses were IgG1 (data not shown). Interestingly, despite the presence of vaccine-induced T cell responses to the HLA-II epitope in patients 1 and 4, no Trojan-specific IgG was detected (figures 4B and 4D). These data demonstrate that the Trojan peptide vaccines are capable of eliciting high titer IgG responses in select patients, suggesting that the peptide-induced CD4 cell responses in these patients are capable of supporting antigen specific CSR.

Antigen specific T cells accumulate at the site of vaccination

In order to further define the nature of the immune response elicited by this vaccine, we next characterized the phenotype and function of cells located at the site of vaccination. Two patients developed palpable inflammatory lesions at the site of immunization (figure 5A). One patient consented to a biopsy of this region (performed 19 days after vaccine # 2), and the inflammatory nature of the response was characterized phenotypically by both immunohistochemistry and flow cytometry and functionally by IFNγ Elispot. Analysis of H&E stained sections revealed granuloma formation, consistent with the well characterized association between Montanide ISA 51 adjuvant and granulomas (24). As demonstrated in figure 5B, the majority of cells recruited to the site of injection were $CD68^+$ monocytes/macrophages, with a smaller population of $CD3^+$ T cells. Subsequent flow cytometric analysis of these cells revealed that the majority of this $CD3^+$ cell population was $CD4^+$, with a limited number of $CD8^+$ T cells (figure 5C). Importantly, from a functional perspective, immune cells obtained from the

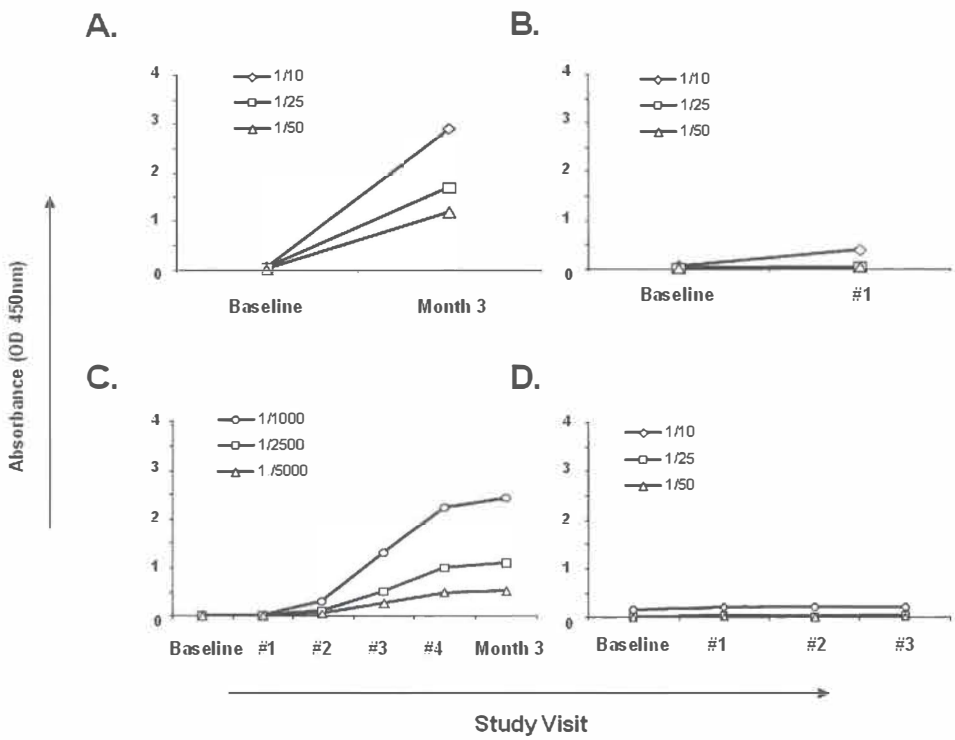

Figure 4. Vaccination with MAGE-A3 and HPV-16 specific Trojan vaccines elicits IgG antibody responses in patients with SCCHN. Trojan-specific IgG was quantified in plasma obtained before immunization and at various time-points after immunization as indicated. MAGE-A3 Trojan (**A+B**) and HPV Trojan (**C+D**) specific IgG was quantified by ELISA. Data represent mean OD readings from triplicate wells derived from three different plasma dilutions.

vaccination site, specifically recognized the HPV-Trojan vaccine ($P<0.0001$) and HPV HLA-II epitope ($P<0.0001$), as determined by an IFNγ re-stimulation Elispot assay (figure 5D). These data demonstrate that vaccination induces ongoing antigen specific immune responses at the site of vaccination.

Antigen specific T cells accumulate within the tumor

In order to understand the nature of the immune response within the tumor microenvironment, we analyzed the cells from an involved lymph node of one patient who underwent a neck dissection post treatment. Importantly, this patient had metastatic neck disease and refused standard of care treatment options prior to vaccination. In order to validate this decision, the patient met with physicians from medical oncology, radiation oncology and otorhinolaryngology-head and neck surgery, each of whom carefully explained the ramifications of this decision to the patient. Furthermore, the patient underwent a psychiatric evaluation and was

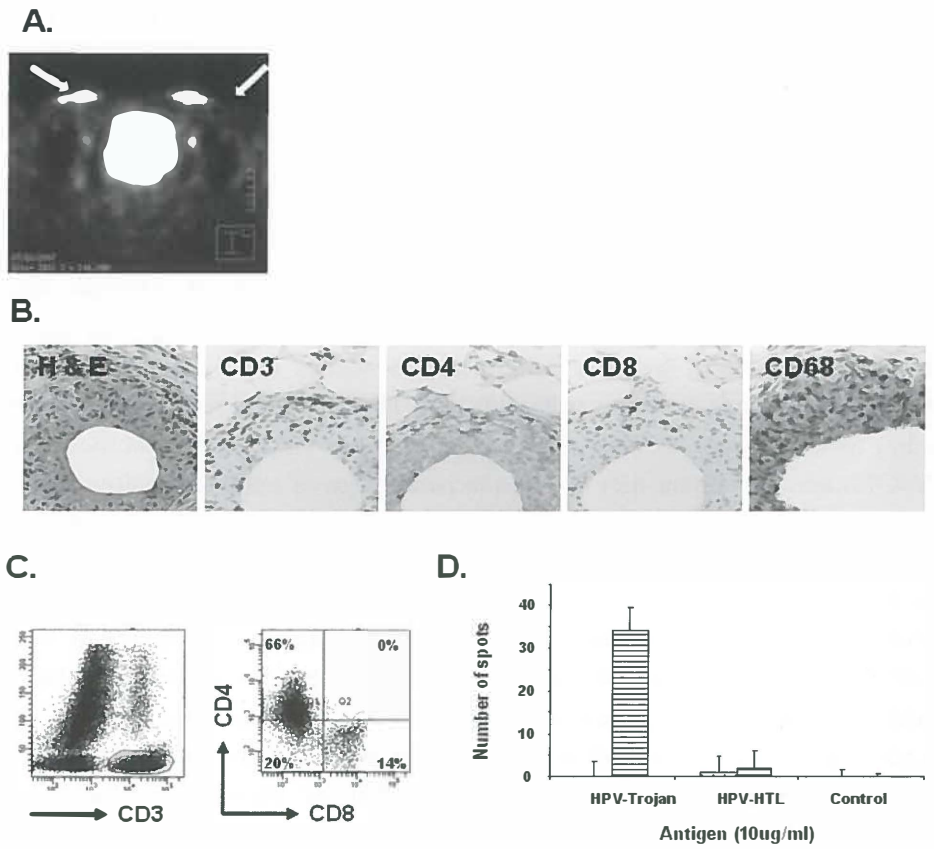

Figure 5. Trojan-specific precursor frequency at the injection site exceeds precursor frequency in the peripheral circulation. Frozen section slides were generated from a granuloma derived from the site of immunization and stained by immunohistochemistry with a variety of leukocyte-specific markers. The granuloma was biopsied 19 days after the second vaccination. PET scan revealing high levels of inflammation at the injection sites as indicated by arrows **(A)**; high numbers of antigen-presenting cells ($CD68^+$) and $CD3^+$ T cells infiltrated the site of immunization **(B)**; part of the granuloma was enzymatically digested into a single cell suspension and evaluated for Trojan-vaccine specificity **(C)**. As measured by flow cytometry, the majority of $CD3^+$ T cells in the granuloma were found to be $CD4^+$ cells. Next, cells were directly *ex vivo* evaluated for Trojan-specificity by IFNγ Elispot. High numbers of Trojan-specific cells were found at the site of injection **(D)**. In contrast, at the same time-interval, no Trojan-specific responses were detected in cells (PBMC) derived from the peripheral circulation. Number of spots per 50,000 cells (granuloma) and 100,000 cells (PBMC) is shown. Error bars represent SD from triplicate wells. * $P<0.05$

judged competent to decline treatment and sign informed consent. Two months after completing the full vaccine protocol, without an objective response as determined by RECIST criteria, the patient agreed to undergo a therapeutic neck dissection. Interestingly, in the pre-treatment biopsy, there was a notable absence of $CD4^+$ T cell infiltration and a limited number of $CD8^+$ cells.

In sharp contrast, in the post treatment biopsy there were abundant $CD4^+$ and $CD8^+$ cells throughout the specimen (figure 6A). Furthermore, there were a qualitatively greater number of apoptotic cells in the post treatment specimen as demonstrated by TUNEL staining. Importantly, these apoptotic cells were judged to be tumor, rather than infiltrating lymphocytes, by our head and neck pathologist (J.P.) who reviewed the slides. Because, it could be argued that these observed phenotypic differences were simply a result of sample error secondary to tumor heterogeneity, we sought to determine the antigen specificity of the TIL. A 48 hour recall experiment demonstrated no significant IFNγ release in response to the peptide antigens and the more sensitive re-stimulation studies could not be accurately completed secondary to poor cell viability. Therefore, we employed tetramer staining as a surrogate to determine the presence of $CD8^+$ cells specific for the HPV-CTL epitope (figure 6B). While the percentage of tetramer positive CD3 T cells in the peripheral blood was 0.4%, the percentage in the lymph node was 8.1%. Interestingly, preliminary studies from this sample revealed that the majority of T cells had a senescent ($CD27^-CD28^-$) phenotype (data not shown) (25). Taken in concert, these data indicate that vaccine-specific immune cells accumulate at the site of immunization and in the tumor micro-environment. Furthermore, the lack of a recall response to the peptides in combination with the presence of $CD27^-CD28^-$ senescent cells within the tumor microenvironment, suggests that the effector function of these antigen specific T cells might be impaired by tumor host interactions.

Discussion

This pilot study tested Trojan peptide-based vaccines in five patients with recurrent or metastatic SCCHN. This novel vaccine contains HLA-I and II restricted peptide epitopes from MAGE-A3 or HPV-16. This study is, to the best of our knowledge, the first to explore therapeutic vaccination with MAGE-A3 and HPV-16 derived HLA-I and II restricted T cell epitopes in recurrent or metastatic SCCHN, and the first to use Trojan constructs for the treatment of human malignancy. Several features of our study are important to consider, both as they relate to the interpretation of our results and to the design of subsequent clinical studies using Trojan peptide vaccines for the treatment of SCCHN.

First, from a clinical perspective, none of the patients developed objective clinical responses by modified RECIST criteria. From a toxicity perspective, the Trojan vaccines were well tolerated and toxicities in general did not require hospitalization. Furthermore, one patient developed significant cerebral edema 24

days after the first vaccination which was associated with hemiplegia and an immune response to both the Trojan and HLA-II restricted peptides. These findings, while clinically intriguing, mandate caution in using Trojan vaccines in patients with known brain metastases.

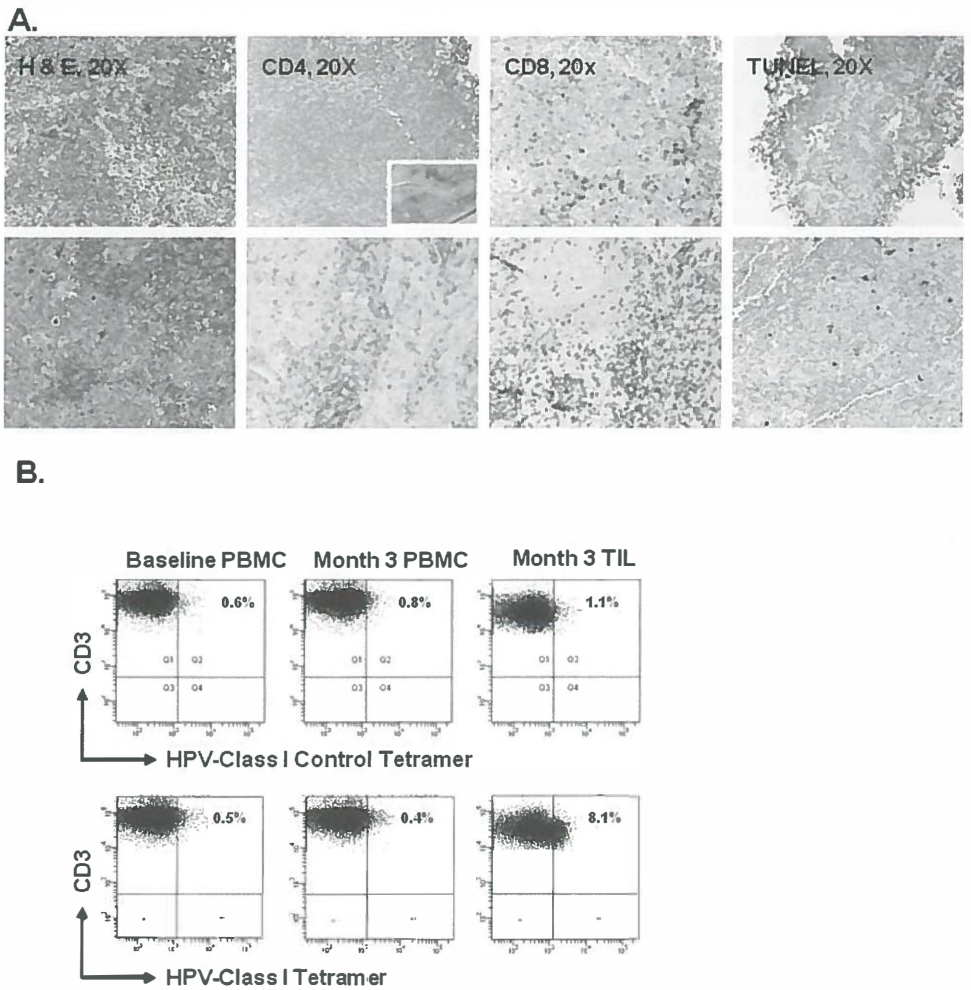

Figure 6. $CD8^+$ and $CD4^+$ T cells infiltrate the tumor microenvironment during immunization and a subset appears to be HPV-specific. Photomicrographs of immunohistochemical staining of tumor tissue harvested before and after immunization with HPV-Trojan vaccine. High numbers of $CD8^+$ and $CD4^+$ cells were found in the tumor micro-environment after immunization **(A)**. Representative sections from CD4 and CD8 staining of the neck metastasis slides before and three months after the last vaccination are shown. Positive controls were positive for all experiments (inset CD4). Part of the neck metastasis, collected at three months after immunization, was enzymatically digested into a single cell suspension (TIL). PBMC collected before and PBMC and TIL collected after three months of immunization were directly *ex vivo* stained with HPV-16 specific HLA-A*0201 tetramer and analyzed by flow cytometry for Trojan HLA-I specificity **(B)**. In comparison to PBMC collected at the same time-interval, TIL derived from the tumor micro-environment after 3 months of immunization were found to be HPV-16 specific, 0.4% and 8.1% respectively.

Second, in four out of five immunized patients, the Trojan-vaccines stimulated systemic T cell responses to both the whole constructs and the constituent HLA-II epitopes. Furthermore, Trojan vaccine-specific IgG antibodies were detected in plasma from two of these patients, suggesting that these CD4 T cells were able to support a humoral immune response. In contrast, the one HLA-I specific IFNγ-producing response was transient. Furthermore, staining of TIL from a tumor resected post-treatment from one patient, demonstrated a significant increase in the number of tetramer positive cells when compared with correlate PBMC. Taken in concert, our data suggest that Trojan peptide vaccines, when administered in combination with Montanide ISA 51 adjuvant and GM-CSF, induce both antigen-specific cellular and humoral immunity, with a biased systemic response to the HLA-II epitopes. Furthermore, although there were no objective responses to the vaccine, it is notable that three patients survived for 24, 27 and 31 (still alive) months post-therapy, in a setting where the average survival is approximately six months. Enthusiasm for these clinical observations must be tempered by small numbers, a potential selection bias, and the fact that these patients received additional therapy after vaccination.

The induction of functional systemic responses to the HLA-II peptides in four out of five patients without durable HLA-I responses in PBMC was surprising. These findings are particularly striking since in three patients, responses to the Trojan constructs were observed in primary recall assays without re- stimulation. Although patients with end stage disease are traditionally considered to be immunosuppressed by virtue of their large tumor burdens, this was not reflected by total white blood cell counts, levels of $CD3^+$ cells, $CD3^+CD8^+$ cells (supplemental table 1) nor the ability to develop Trojan-specific immune responses (table 3).

While it is possible that these responses were predominated by the presence of regulatory T cells (Tregs), we do not think this is likely. Specifically, the Elispot assays used IFNγ as a read out. While Tregs are recognized to be induced by this cytokine, they do not generally produce IFNγ (26). Furthermore, although Tregs suppress CSR (24), two of the treated patients developed IgG antibody responses to the Trojan peptides. Thus, our findings demonstrate that this vaccine strategy may elicit functional cellular and humoral systemic immune responses to both the entire Trojan constructs and their constituent CD4 restricted epitopes. These observations are particularly relevant in light of a recent report demonstrating that adoptive transfer of antigen-specific CD4 cells can mediate regression of metastatic

melanoma (27) and animal studies showing that antigen-specific CD4 cells can effectively mediate direct regression of HLA-II positive melanomas (28, 29).

Several possible explanations may account for the lack of durable HLA-I responses in our study. First, recent data suggests that patients with SCCHN have a high frequency of HLA-II precursors to MAGE-A3 in the absence of prior vaccination, suggesting that this cell population may be readily responsive to peptide stimulation (30). In addition, since SCCHN are recognized to escape the immune response through deletion/anergy of HLA-I type effectors through Fas-FasLigand (Fas-FasL) interactions, it is likely that a limited number of antigen-specific precursors are available for subsequent expansion (31). Second, two of the four HLA*A02 positive patients were determined to be HLA*A0205 and HLA*A0206, respectively. Although data is limited, it is thought that HLA-I epitope recognition is reduced in these patients, potentially influencing the development of HLA-1 restricted T cell immunity (32). Finally, it is now well established that HLA-I immune responses are more prevalent in lymph nodes, suggesting that our decision to study PBMC may have biased our outcome (33). Further bias may have also been introduced in our experimental design, where we re-stimulated *in vitro* with the Trojan vaccine, rather than the individual epitopes.

Interestingly, in the one patient from whom we were able to compare pre-and post treatment biopsies, there was a profound increase in both $CD4^+$ and $CD8^+$ T cell responses at three months following vaccination. Furthermore, TIL from this patient demonstrated a high percentage of antigen-specific T cells in the post-vaccination biopsy. Importantly, the fact that these TIL did not secrete IFNγ in a recall assay and demonstrated a largely $CD27^-CD28^-$ phenotype (data not shown), suggests that they were biologically exhausted or senescent (25). This idea is consistent with previous observations that $CD27^+/CD28^+$ T cells are responsible for the therapeutic effects in melanoma patients responding to adoptive T cell transfer (34). However, data from colon cancer patients, demonstrate that the presence of both CD27/CD28 double negative and double positive CD8 cells in the tumor microenvironment are independently associated with reduced metastases (35). Further studies will be required to definitively characterize the function of these antigen-specific T cells.

Two of the major difficulties experienced within patient accrual in this pilot study included the unexpected low frequency of HLA-A*02 expression among this patient population and relatively quick deterioration of most recurrent or metastatic

SCCHN patients. In fact, HLA-A*02 is frequent in all ethnic groups and is found in approximately 35% and 50% of African-Americans and Caucasians, respectively (36). Surprisingly, only 20% of African-Americans and 38% of Caucasians were found to be HLA-A*02 positive in our total study population (table 2).

Interestingly, a clear increase in MAGE-A3 HLA-II specific T cells was observed in one study patient who was determined to express HLA-DR*15. Although HLA-DR*04 and HLA-DR*07 specificity for the MAGE-HTL epitope, incorporated in our MAGE-Trojan vaccine, has previously been reported (16), it suggests that this MAGE HLA-II epitope is promiscuous and immunogenic in patients who are not DR*04 and/or DR*07. Based on the low percentage of HLA-A*02 expression in this population, the limited tolerance of these patients to nearly four months of vaccine delivery and the induction of functional responses to both the Trojan vaccine and the HLA-II restricted epitopes, we are currently conducting a phase I clinical trial in which patients are immunized every two weeks and in which HLA-A*02 is not required for study entry. We anticipate that these changes will increase the feasibility of our approach without inhibiting efficacy.

In summary, this pilot study demonstrates the feasibility of using Trojan peptide vaccines in combination with Montanide ISA 51 adjuvant and GM-CSF for the treatment of patients with advanced SCCHN. These data add to a growing body of literature demonstrating the efficiency of using long peptides to prime antitumor immune responses (11). The fact that these Trojan constructs were effective in stimulating responses against both the parental vaccine as well as its HLA-II restricted epitopes, limits traditional feasibility concerns associated with the use of HLA-I restricted peptides. Furthermore, while our conclusions must be viewed in the context of limited sample size, the presence of antigen-specific cells both at the vaccine site and within the tumor microenvironment, suggests that these Trojan peptide-primed cells may home to the tumor site. From a clinical perspective, the ability of this vaccine strategy to induce humoral and cellular responses with limited toxicity, may allow it to serve as a solid foundation on which to add additional immuno-modulators. Alternatively, these Trojan peptides might be effective as "mono-therapy" to limit the risk of disease recurrence following standard therapy for patients with high risk SCCHN. Our ongoing studies are exploring both of these options as a means to both limit the treatment related morbidity and prolong the survival of our patients afflicted with SCCHN.

Acknowledgement

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8

Summary, discussion and future perspectives

Conventional cancer therapies are valued for their antitumor effects. However, they lack tumor specificity, may reverse antitumor immunity (e.g. by causing inflammation) and generally do not induce protective antitumor immune responses. Cancer immunotherapy is thought to facilitate long-lasting antitumor immune responses, which induce tumor regression and protection against recurrence. Since the discovery of naturally occurring cell-mediated immune responses in melanoma patients in the mid-1970s, and since the cloning of the first tumor-associated antigen in the mid-1990s, various strategies designed to induce or boost antitumor immunity have been developed (1,2). Currently, a total of eight immunotherapeutics have been approved for the treatment of solid malignancies and several others are currently under evaluation in various clinical trials. Despite these promising advances, cancer still contributed towards 571,950 deaths in the USA in 2011 (3) and to date, no cancer-specific immunotherapeutic agent used alone can permanently cure patients suffering from solid malignancies. Increasing evidence suggests that only the combined use of various immunotherapeutics can induce long-lasting, protective tumor regression and overcome tumor escape from immunosurveillance and immunosuppression.

This thesis focuses on CD137-mediated co-stimulation **(part A)** and NK cell-based immunotherapy **(part B)** and their therapeutic potential in the treatment of patients with solid malignancies. Fundamental for NK cell-based immunotherapy is a sufficient *ex vivo* expansion of large numbers of cytotoxic NK cells which has previously been shown to be CD137-CD137L mediated (4). Therapeutic potential, challenges in clinical translation and possibilities for their combined use are discussed. In addition, results of a pilot study testing a MAGE and HPV peptide-based vaccine are presented and discussed in the clinical perspective of the aforementioned immunotherapeutic strategies **(part C)**. Overall, therapeutic strategies discussed in this thesis provide a foundation on which to add additional immunotherapies to facilitate improved treatment of select solid tumors.

CD137 mediated co-stimulation: Lessons learned and hurdles to overcome

Based on the therapeutic potential of anti-CD137 mAbs for the treatment of cancer and autoimmunity (5-9), the demonstrated importance of NK cells in CD137 mediated antitumor responses in murine models (6,10) and the recognized importance of Fc-FcγR interactions on mAb function (11,12), we evaluated in **chapter 2** the ability of two different glycoforms of a chimerized anti-human CD137 mAb, an aglycosylated (GA) and a low fucose form (GG), to react with

human NK cells. Because of the recognized role of NK cells in the antitumor function of anti-CD137 mAb in murine models, we initially hypothesized that IL-2 stimulated human NK cells would express CD137 and that ligation with chimeric anti-CD137 mAb would result in cytokine release and degranulation. Surprisingly, we found that, unlike their murine counterparts, human NK cells do not express high levels of CD137 following IL-2 stimulation and subsequent culture. In contrast, CD137 is stimulated on IL-2 activated human NK cells following culture in the presence of immobilized glycosylated mAb or papain cleaved Fc fragments. The inability to stimulate CD137 expression was independent of the antigen specificity of the Fab region and the magnitude of CD137 expression was associated with patterns of glycosylation known to enhance Fc-FcγR interactions. In addition, Fc dependent CD137 expression was associated with cell activation as demonstrated by enhanced CD69 and CD54 expression and the release of the pro-inflammatory cytokines IFN-γ and TNF-α. Furthermore, CD137 expression was preceded by degranulation which results in reduced cytotoxicity of CD137 expressing NK cells against NK sensitive tumor targets.

The unexpected findings in **chapter 2** raised concerns whether murine-based animal models would accurately predict human immune responses to CD137 manipulation. Limited data exists on B cell associated CD137. This is surprising since their importance in antitumor immune-regulation and autoimmunity is well documented (13,14) and CD137 mediated T cell immunomodulation has been postulated to be mechanistically responsible for observed changes in B cell function, including diminished isotype-specific antibody responses and changes in B cell survival (15-17). Importantly, murine B cells do not express CD137, while human B cells are reported to up-regulate CD137 in response to anti-IgM stimulation (18-20) Therefore, in **chapter 3**, we performed *in vitro* studies to elucidate, which external signals regulated CD137 expression on human B cells and defined the biological effect of CD137-mediated co-stimulation on human B cells. Our studies revealed that despite the absence of CD137 on murine B cells, CD137 is expressed on immunoglobulin (Ig)-activated human B cells following B cell receptor (BCR) stimulation. Cognate help from T cells through CD40-CD40L interaction and cytokines are mandated since anti-CD40 stimulation or cytokine alone were not capable of inducing CD137 expression in the absence of BCR stimulation. These findings suggest that CD137 expression on human B cells is tightly controlled and strictly dependent upon antigen encounter while the BCR serves as the initial "switch", which enables CD137 expression on the B cell surface. Importantly, CD137 ligation stimulated B cell proliferation, enhanced B

cell survival and induced TNF-α and TNF-β secretion. Furthermore, CD137 expression was preceded by CD137L expression when assessed *in vitro*. In addition, both $CD137^+$ and $CD137L^+$ B cells were detected *ex vivo* in human tonsils, although receptor and ligand are primarily expressed on distinct populations.

Events such as the TGN1412 trial in 2006, where healthy volunteers became critically ill following the first in-human administration of a super-agonistic anti-CD28 mAb (21), underscore the importance of appropriate preclinical testing *in vitro* in human PBMC and *in vivo* in mice and non-human primate models. The disparity of CD137 expression and function between human and murine NK and B cells challenges the use of murine-based disease models for the evaluation of CD137-mediated immune regulation and toxicity. To facilitate proper animal choice in preclinical animal testing, we performed binding studies of the chimeric glycosylated anti-CD137 mAb (GG) against activated PBMC from cynomolgus macaque and baboon (**chapter 4**). Surprisingly, the chimeric glycosylated anti-CD137 mAb only bound human CD137, while a commercial mouse anti-human CD137-specific mAb bound activated CD137-expressing PBMC from both human and non-human primates. In order to understand the differences in binding affinity between these mAbs, we sequenced cynomolgus macaque and baboon CD137 specific cDNA and found that the amino acid sequence of CD137 is largely conserved between human and primate species, with the extracellular domain differing by only 9-10 amino acids. Glycosylation seemed not responsible for the observed binding differences since two potential amino acids highly suggestive for glycosylation (Asn^{103} and Asn^{115}) did not differ between species. Subsequent replacement of cynomolgus macaque CD137 sequences with human CD137 sequences identified the three amino acids Thr^{66}, Pro^{67} and Phe^{69} as being critical for GG binding. In addition, these amino acids are likely involved in a conformational rather than a linear epitope, as an overlapping peptide was unable to inhibit GG recognition, even at excess molar concentrations.

The findings in **chapter 2** provide important insight into the design of antibodies intended to manipulate co-stimulatory pathways for clinical benefit. Although our studies did not elucidate if similar findings would apply for other co-stimulatory molecules, Maniar et al. recently published enhanced expression of OX40 on human NK cells in the presence of immobilized human IgG1, whereas IgG1-mediated expression of CD28, CTLA-4, ICOS and PD-1 was absent (22). The data presented in **chapter 2** also support the idea that CD137 ligation may co-stimulate

antitumor function of Fc-primed NK cells. This hypothesis is supported by previous observations that human NK cells transfected with a construct containing the *intracellular* domain of CD137 directly linked to an anti-CD3/CD19 chimeric receptor were more effective than NK cells transfected with an anti-CD3/CD19 chimeric receptor alone, in both killing leukemic cells and producing IFN-γ and GM-CSF (4). Additional studies by maniar et al. confirmed the notion that mAbs, which target tumor-associated antigens, when composed of the appropriate Fc, can also provide a first signal for NK cell-dependent CD137 expression since cocultures of human NK cells with mAb-opsonized head and neck cancer-derived tumor cells enhanced CD137 expression on human NK cells *in vitro*. Moreover, recent studies by Kohrt et al. demonstrated that sequential administration of anti-CD20 and anti-HER-2/neu mAb followed by anti-CD137 mAb mediated co-stimulation enhances NK cell-mediated cytotoxicity against lymphoma and breast cancer cells in murine models (23,24). Overall, the findings in **chapter 2** shed new light onto a secondary function of Fc-FcγRIII ligation and demand caution when describing CD137-specific mAbs as being "agonistic" if their function is assessed with intact Fc regions. Particularly, effects previously attributed to CD137 interactions with the Fab region, must now be re-evaluated as potentially related to Fc induced expression of CD137 on NK cells with subsequent effects due to (i) direct interaction of CD137L with Fc induced CD137, (ii) direct stimulation of Fc induced CD137 by agonistic anti-CD137 mAbs or (iii) antibody blockade of interactions between Fc induced CD137 and CD137L. In addition, the findings in **chapter 2 and 3** have important implications for the clinical translation of CD137-based immunotherapeutic strategies. The disparity of CD137 expression and function between human and murine NK and B cells challenges the use of murine-based disease models for the evaluation of CD137-mediated immune regulation. As a result, targeting the CD137 pathway with therapeutic intent may have unanticipated consequences on human B and NK cell function.

Importantly, CD137 is recognized for the treatment of both cancer and autoimmunity (5-9). This dual function should be taken into account when analyzing the direct effect of anti-CD137 targeting immunotherapeutics in clinical cancer studies. Specifically, CD137-mediated B cell proliferation and survival are likely to positively affect antitumor immunity. However, CD137 stimulation greatly enhanced TNF-α and TNF-β secretion by anti-Ig/anti-CD40 activated B cells. These cytokines independently or complementarily enhance immune responses and augment inflammation by targeting various immune cells which may negatively affect antitumor immune responses. TNF-α and TNF-β act both

stimulatory and inhibitory depending on the site and time of activation. It has been shown to be important in the early phase of inflammation (25,26) which promotes tumor cell growth, survival and angiogenesis (27). In contrast, failure to regulate TNF production induces chronic inflammation which may inhibit immune responses (28). In addition, TNF-α has been shown to play a role in cytokine-induced apoptosis of NK cells (29). Only outcome analysis of phase I/II clinical studies can define which function will be more prominent. Finally, Fc stimulated NK cells did not express CD137L. However, B cells were found to express CD137L during the early stages of B cell activation followed by the up-regulation of CD137. This may suggest that reverse signaling plays a role in B cell function, since CD137L has been shown to also reverse signal in other cell types (30,31).

Safe and effective translation of antibodies designed to manipulate co-signaling pathways requires in depth preclinical non-human *in vivo* assessment. The translational relevance of such *in vivo* studies is largely dependent upon the ability of the study drug to elicit similar functional responses in the animal chosen. The findings presented in **chapter 4** underscore the importance of careful animal selection before preclinical *in vivo* testing is initiated. This became clear by the in depth *in vitro* studies performed shortly after the TGN1412 trial. The extensive cytokine release in this in-human trial was not foreseen by thorough *in vitro* (human PBMC) and *in vivo* (mice and cynomolgus macaque) preclinical studies which excluded differences in the extracellular domain, intracellular domain and binding affinity of cynomolgus macaque CD28 (32). However, post-clinical re-evaluation demonstrated various differences in CD28-mediated immune reactivity between human and cynomolgus macaque PBMC (33). In particular, murine Treg were found to efficiently prevent CD28-triggered cytokine production (34) and cynomolgus macaque CD4+ T cells loose CD28 expression during differentiation into effector T cells, in contrast to human CD4+ T cells, which maintain CD28 expression (35). Ideally, safety studies of the presented CD137 antibodies should be performed in chimpanzees since this animal is the only animal that possesses Thr^{66}, Pro^{67} and Phe^{69} responsible for GG binding. However, research using chimpanzees is profoundly restricted. In addition, inherent polymorphisms for CD137 within individuals of a species should be taken into account since they have been reported in cynomolgus macaques (36). In this regard, only in-human studies will provide definitive proof of safety.

NK cell-based immunotherapy in patients with solid malignancies: Lessons learned and hurdles to overcome

The pivotal role of NK cells in CD137 mediated antitumor immunity, the reported success of NK cell-based immunotherapy in patients affected by hematological malignancies and the importance of CD137-CD137L ligation in *ex vivo* NK cell expansion formed the foundation of the studies presented in **chapter 5**. This chapter extends the results presented in **chapter 2**. We found that NK cells from normal individuals and patients affected by gastric cancer, lung cancer, colon cancer and hepatocellular cancer rapidly expand *ex vivo* from PBMC in the presence of exogenous IL-2 and K562 cells expressing membrane-bound IL-15 and CD137L. Based on CD3 and/or CD56 phenotype, the majority of cells in the expanded cell product represented NK cells while a much smaller proportion represented NKT and T cells. *Ex vivo* expansion tended to alter the balance of NK cell receptor expression towards those that activate and mediate cytotoxicity. Additional studies confirmed their lytic activity, both directly and indirectly through ADCC, against autologous tumor targets while sparing autologous PBMC. Immunomagnetic bead separation confirmed that little to no cytotoxictity was mediated by the T and NKT cell population. To facilitate clinical translation, we also confirmed that NK cells can be expanded from lymphocyte-enriched cell fractions obtained from PBMC by GMP compliant counter current elutriation and that these NK cells are able to lyse allogeneic prostate-derived tumor cell lines in a similar fashion.

The interaction of Fc fragments of antibodies with Fcγ receptors is an essential checkpoint in ADCC. Specific polymorphisms at amino acid position 158 enhance FcγRIII affinity for IgG1 and are associated with improved clinical outcome in lymphoma patients treated with anti-CD20 antibody (12). Based on the previous findings that (i) patterns of Fc glycosylation known to impact Fc interactions with FcγRIII directly affect CD137 expression and NK cell activation (**chapter 2**) and (ii) that the cytolytic capacity of expanded autologous NK cells is enhanced in the presence of the chimeric anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab (**chapter 5**), we extended our studies to define the importance of FcγRIII polymorphisms in cetuximab-mediated ADCC and used various head and neck derived tumor targets as an *in vitro* study model in **chapter 6.** Polymorphism frequency at amino acid 158 from 45 healthy individuals was 51.1% for *F/F*, 37.8% for *V/F* and 11.1% for *V/V*. ADCC assays against three different head and neck carcinoma cell lines confirmed that cetuximab-mediated ADCC *in vitro* is enhanced in individuals possessing at least one *V* allele. In

addition, we found evidence that NK cells containing a single *V* allele mediate better direct cytotoxicity in an antibody-independent manner since donor NK cells possessing at least one *V* allele showed significantly enhanced cytotoxicity against NK sensitive K562 targets. These findings have significant implications for the identification of solid tumor patients, including head and neck cancer patients, which are most likely to respond to anti-EGFR antibodies and/or NK cell-based immunotherapy.

The use of NK cells as a treatment modality in solid tumors requires that large numbers of NK cells are generated that kill tumor cells directly or augment the cytotoxic effect of tumor directed monoclonal antibodies. In general, NK cell mediated cytotoxicity requires HLA class I and KIR mismatching to overcome natural inhibition. For that reasons, allogeneic NK cells are postulated to be more cytotoxic than their autologous counterparts. For clinical translation of allogeneic treatment protocols in solid malignancies, cell products have to contain pure NK cell populations free of contaminating T and NKT cells in order to prevent graft-versus-host disease (GvHD) (37). Although unwanted allogeneic T and NKT cells can now be depleted by human-grade and GMP conform magnetic bead procedures, single contaminating T and NKT cells may still persist. Depletion of contaminating T and NK cells is likely to be less critical in an autologous treatment setting. Under the *ex vivo* expansion conditions presented in **chapter 5**, NK cells up-regulated various receptors associated with activation and cytotoxicity. This resulted in natural cytotoxicity against autologous gastric tumor cells partially mediated through NKp46 and NKp30. In general, data on NK cell mediated autologous tumor cell killing in solid malignancies is limited, although autologous NK cell mediated cytotoxicity has recently been shown in melanoma (38).

To facilitate clinical translation, current clinical cellular therapy protocols were adapted to explore the possibility to serve in future NK cell-based immunotherapy studies. The Elutra® cell separator uses counter-flow elutriation technology to separate PBMC primarily by size and secondarily by specific gravity. This generates five separate cell fractions including platelets (fraction 1), erythrocytes mixed with lymphocytes (fractions 2), lymphocytes (fractions 3), lymphocytes mixed with monocytes (fraction 4) and monocytes (fraction 5). Currently, monocytes obtained from fraction 5 are used to generate dendritic cells for cancer immunotherapy while the cells from fractions 2, 3 and 4 are usually "archived" in liquid nitrogen. Studies in **chapter 5** confirm that NK cells could also be expanded from elutriated cell fractions. Although variability in expansion rates between

healthy individuals was observed, these data provide a foundation for the large-scale production of cytolytic NK cells from elutriated cell fractions. Expanded NK cells could be employed alone or in combination with other cellular components such as DC generated by fraction 5 for application in cellular therapy of cancer.

Importantly, we demonstrated that the receptor-ligand pairs mediating autologous NK cell toxicity is patient dependent. These differences likely reflect variation in expression of receptor-ligand combinations in human that are known to be operative in the control of NK cell cytotoxicity. These variations include HLA and KIR polymorphisms as well as tumor type and tumor origin (e.g. primary versus metastatic tumor cells; lymph node metastases versus organ metastases) (38,39). Instruments to easily identify patients who are likely to have optimal cytotoxic receptor-ligand combinations are lacking and this should be taken into account when data from phase I toxicity trials is interpreted in terms of clinical benefit. The finding that *ex vivo* expanded NK cells mediate ADCC, which significantly improved overall NK cell-mediated cytotoxicity, implicates that the combined strategy of adoptively transferred *ex vivo* expanded NK cells with concurrent infusion of a mAb that is approved for cancer immunotherapy may provide clinical benefit for the treatment of select solid tumors. Enhancement in cytotoxicity was far more dramatic in the autologous setting if compared to the allogeneic setting suggesting that Fc receptor ligation augments ongoing receptor-ligand interactions or alternatively, triggers additional receptor-ligand interactions associated with natural cytotoxicity. Importantly, efficient tumor cell killing is highly dependent on the potential of adoptively transferred NK cells to home and infiltrate into solid tumor tissue. This may be directed by opsonizing tumor cells with a cancer cell directed mAb although clinical studies are needed to confirm this hypothesis.

Moreover, the findings presented in **chapter 6**, confirm the impact of Fc polymorphisms in ADCC against EGFR expressing tumor targets. Careful pre-entry patient selection is likely to enhance clinical outcome in future phase I/II clinical trials testing tumor-specific mAb in solid tumor patients. In addition, Fc polymorphism screening may prove useful in defining the patients, who are likely to respond to treatment with currently approved tumor-specific mAbs. Importantly, effects of tumor-antigen density, also reported to affect EGFR-specific ADCC (40), were not taken into account in the studies presented in **chapter 6**.

Scientific possibility and clinical reality: Lessons learned and hurdles to overcome

Chapter 7 summarizes a clinical study on the safety and immunogenicity of a MAGE-A3 and HPV-16 peptide-based vaccine for the treatment of head and neck cancer patients. Both vaccines were composed of HLA class I and HLA class II restricted MAGE-A3 or HPV-16 derived peptides, joined by furin-cleavable linkers which enable release of the individual peptide-epitopes in the Golgi where furin endopeptidase resides (41). In addition, a "penetrin" peptide sequence derived from HIV-TAT was added, which allows the entire peptide-vaccine to translocate through the cell membrane and penetrate directly into the ER and Golgi where they can form peptide-HLA complexes (42). In this clinical study, we screened 31 patients with recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN) and five patients were treated. Three patients received the MAGE-A3 peptide vaccine composed of two HLA class I epitopes and one HLA class II epitope and two patients received the HPV-16 peptide vaccine composed of one HLA class I epitope and one HLA class II epitope. In four out of five immunized patients, the Trojan-vaccines stimulated systemic T cell responses to both the whole construct and the constituent HLA class II epitopes and Trojan vaccine-specific IgG antibodies were detected in plasma from two of these patients. In addition, one transient HLA class I specific immune response was observed and staining of tumor-infiltrating lymphocytes (TIL) from a tumor resected post-treatment from one patient demonstrated a significant increase in the number of vaccine-specific HLA class I restricted T cells when compared with correlate PBMC. Furthermore, vaccine-specific HLA class II specific T cells were found at the site of immunization. Importantly, from a toxicity perspective, the Trojan vaccines were well tolerated and toxicities in general did not require hospitalization. One patient with a known brain metastasis developed significant cerebral edema 24 days after first vaccination with the Mage-Trojan vaccine, which was associated with hemiplegia and an immune response to both the whole construct and HLA class II restricted peptides. In addition, from a clinical perspective, none of the patients developed clinical responses by modified RECIST criteria.

Several features of the Trojan pilot study are important to consider, both as they relate to the design of clinical studies in general and to the design of subsequent clinical studies using Trojan vaccines for the treatment of SCCHN. First, the lack of induction of durable HLA class I responses was surprising, yet explainable. Recent data suggests that cancer patients possess HLA class II precursors to

MAGE-A3 in the absence of prior vaccination (43), suggesting that this cell population may readily respond to peptide stimulation. In addition, immune escape through deletion/anergy of HLA class I effector T cells through Fas-FasL interactions may result in limited numbers of antigen-specific precursors available for subsequent stimulation. Furthermore, two of the four HLA*A02^{+} patients were determined to be HLA*0205 and HLA*A0206, respectively. These HLA*A02 epitopes are associated with reduced HLA class I restricted T cell immunity (44). Finally, tumor specific immune responses are more prevalent in lymph nodes (45), suggesting that the immunomonitoring studies using PBMC may have biased immunological outcome.

Importantly, despite the lack of CTL responses, T helper cell responses were elicited even in patients with end-stage disease, who are traditionally considered immunosuppressed by virtue of their large tumor burdens. Moreover, the stimulation of T helper cell responses is particularly relevant in light of a recent report demonstrating that adoptive transfer of antigen-specific T helper cells can mediate regression of metastatic melanoma (46) and animal studies showing that antigen-specific T helper cells can effectively mediate direct regression of HLA class II positive melanoma (47,48).

Two of the major hurdles experienced in this pilot study relate to patient accrual. These included the unexpected low frequency of HLA-A*02 expression among this patient population and the relatively quick deterioration of clinical performance status. HLA-A*02 is frequent in all ethnic groups and is found in approximately 35% and 50% of African-Americans and Caucasians, respectively (49). Among our patients, only 20% of African-Americans and 38% of Caucasians were found to be HLA-A*02^{+}. Further studies in a larger patient cohort are needed to confirm this finding which implies that the HLA-A*02 allele might be of prognostic relevance in SCCHN.

Although there were no objective (RECIST) responses to the vaccine, it is notable that three patients survived for 24, 27 and 31 months post-therapy, in a setting were the average survival is approximately six months. The ability of the Trojan vaccines to induce humoral and cellular responses with limited toxicity, may allow them to serve as a solid foundation on which to add additional immune-modulators such as CD137 mAb. Alternatively, the Trojan vaccines might be effective as monotherapy to limit the risk of disease recurrence following standard therapy for patients with high risk SCCHN.

Future perspectives

In the past 20 years, immunotherapy has found its place into the daily treatment of patients suffering from various hematological and solid malignancies. Progress in the understanding of its mode of action has accelerated clinical interest and resulted in numerous ongoing immunotherapy-based clinical studies targeting various tumor-specific antigens (www.clinicaltrials.gov). In addition, immunotherapeutic strategies were extended towards the direct manipulation of immune-modulatory pathways. First clinical success of an immune-modulatory immunotherapy was achieved almost a year ago when a CTLA-4 blocking mAb, ipilimumab, demonstrated effective in enhancing overall survival in metastatic melanoma (50). This thesis provided various approaches of cancer immunotherapy which can be used alone, in combination, or in conjunction with currently approved treatment modalities. Several findings in this thesis justify further investigation.

First, CD137 ligation has been shown to promote both T cell (51) and B cell (this thesis; (52)) proliferation and survival. Therefore, studies that further elucidate the functional relevance of CD137 expression on human NK cells are highly needed. If proven equal, their *in vivo* persistence after adoptive transfer may be enhanced through CD137 targeting. Furthermore, activated NK cells up-regulate HLA class II and co-stimulatory molecules (unpublished data) and their ability to internalize, process and present influenza-derived antigens to $CD4^+$ T cells has been described (53). Extended studies are of clinical interest to define if T cell responses directed against tumor cells are optimized by NK cell mediated antigen presentation.

Second, the concurrent expansion of NKT cells from PBMC during *ex vivo* NK cell expansion sets the stage for their clinical translation into new cellular therapy protocols. Specifically, $\gamma\delta$-TCR^+ T cells possess strong lytic potential after activation with isopentenyl pyrophosphate (IPP) and IL-2 (54) and they have been shown to enhance NK cell mediated cytotoxicity through CD137-CD137L interactions (22). Infusion of $\gamma\delta$-TCR^+ T cells alone or in combination with NK cells in newly proposed immunotherapy protocols may induce favorable clinical responses. Also, the preliminary findings that Fc polymorphisms may also play a role in natural cytotoxicity should be further studied in order to clarify the direct association of a *V* allele with enhanced expression of natural cytotoxicity and activating receptors. If confirmed, stringent patient selection will become possible in future clinical studies testing adoptive NK and/or $\gamma\delta$-TCR^+ T cells transfers or tumor antigen-specific mAb which in turn, may positively affect clinical response rates and select for patients who benefit the most. However, optimism should be

dampened since a *V/V* phenotype is relatively rare in the general population (this thesis; (12,40,55)). This may impact patient accrual rate and should be taken into account when proposing a clinical trial. In addition, some combined strategies may not prove to be optimal since the addition of a gp100 peptide vaccine to ipilimumab treatment increased the risk of tumor progression and shortened tumor responses (56).

Finally, immunotherapy-induced clinical benefit requires the induction of an equilibrium in which tumor-specific immune cells constantly overrule or at least equal tumor cell growth. To establish such an equilibrium is likely to require less time in patients who are tumor free or have minimal residual disease, compared to patients with large tumor burdens. Therefore, tumor mass reduction through surgery and/or radiation should be considered as a complementary entry criteria in studies evaluating immunotherapeutic strategies in patients with metastatic disease who failed therapy with approved treatments. This could reduce the number of patients who have to be excluded from study protocols due to rapid disease progression. In addition, the approval of ipilimumab demonstrated the insufficiency of conventional RECIST to evaluate clinical success in terms of objective tumor responses, which led to the incorporation of immune-related response criteria (irRC). These response criteria should now be extended and define an equilibrium (e.g. stable disease) as a positive responses criteria. Importantly, applying irRC on previously ran clinical studies will likely re-identify various immunotherapeutic strategies with proven favorable toxicity profiles and potential clinical benefit for patients with solid tumors. It might be more cost-effective to first reconsider the potential of these previously tested options before proposing to test others.

In conclusion, the field of immunotherapy is rapidly expanding treatment possibilities for patients with malignancies. These treatments require a more "personalized medicine" approach and novel evaluation criteria. A combination of therapy options is probably the most effective and could exist of vaccination, adoptive cell transfer and modulation of the response by therapeutic antibodies.

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9

Summary in dutch
Nederlandse samenvatting

Achtergrond

In het dagelijks leven staan we continu bloot aan potentiële ziekteverwekkers, zoals bacteriën en virussen. Ons lichaam wordt beschermd tegen deze ziekteverwekkers door een leger aan witte bloedcellen die gezamenlijk het afweersysteem, ook wel immuunsysteem genoemd, vormen. Het afweersysteem zorgt ervoor dat alles wat niet in ons lichaam thuis hoort, wordt herkend en opgeruimd. Het bestaat uit twee verschillende delen. Het eerste deel, het aangeboren afweersysteem, wordt gevormd door neutrofielen, macrofagen, natural killer (NK) cellen, γδ T cellen, monocyten en dendritische cellen. Het aangeboren afweersysteem reageert direct na het binnendringen van een ziekteverwekker, waarbij de afweerreactie niet specifiek en slechts van korte duur is. Echter, het aangeboren afweersysteem activeert het tweede deel van ons afweersysteem, het verworven afweersysteem. Dit deel komt langzaam op gang, herkent specifiek de lichaamsbedreigende aanvaller en beschermt voor langere tijd tegen de betreffende ziekteverwekker. Het verworven afweersysteem wordt gevormd door T cellen en B cellen. Als een gezonde cel geïnfecteerd wordt door een ziekteverwekker worden delen van de ziekteverwekker (peptiden) via de HLA moleculen op het celoppervlak van de geïnfecteerde cel aan het afweersysteem gepresenteerd. Hierdoor wordt de aangeboren en verworven afweer op gang gebracht. Op het moment dat een gezonde cel in een tumorcel verandert, worden delen van het tumorunieke materiaal (tumorspecifieke peptiden) ook via de HLA moleculen op het celoppervlak van de tumorcel aan het afweersysteem gepresenteerd. Dit betekent dat in principe ook kankercellen door het afweersysteem kunnen worden herkend en opgeruimd. Immuuntherapie bij kanker (tumorspecifieke immuuntherapie) is een behandeling die erop is gericht het afweersysteem te stimuleren om tumorcellen te herkennen en op te ruimen.

Aangeboren afweer

NK cellen

Binnen de aangeboren afweer spelen NK cellen een belangrijke rol in de herkenning en vernietiging van tumorcellen. Hun functie wordt gereguleerd door een complex netwerk van celstructuren (receptoren) welke diverse "tegenstructuren" (ligands) op een tumorcel kunnen herkennen. De interactie tussen een receptor en zijn specifieke ligand resulteert in een afweer remmende reactie als het gaat om een gezonde lichaamseigen cel, welke niet opgeruimd hoeft te worden. Daarentegen resulteert de interactie tussen een receptor en zijn specifieke ligand in een afweer activerende (cytotoxische) reactie als het om een geïnfecteerde cel of een tumorcel gaat, welke opgeruimd moet worden.

Dendritische cellen

Dendritische cellen bezitten de unieke eigenschap dat ze kankerspecifieke eiwitstructuren (antigenen) opnemen en in brokjes (peptiden) presenteren aan T cellen. De presentatie van de opgenomen peptiden vindt voornamelijk in de lymfklieren plaats, via zogenaamde HLA moleculen. Dit zijn speciale presentatiemoleculen welke zich op bijna alle cellen in het lichaam bevinden. Er worden twee soorten presentatiemoleculen onderscheiden; HLA klasse I en II moleculen. Ook tumorcellen bezitten HLA moleculen, voornamelijk HLA klasse I moleculen. Tot nu toe is aangenomen dat vooral de HLA klasse I moleculen van belang zijn voor de afweer tegen tumorcellen.

Verworven afweer

Ten grondslag aan de verworven afweer tegen tumorcellen ligt de herkenning van peptiden afkomstig van tumorspecifieke eiwitten die via HLA-moleculen op het cel oppervlak gepresenteerd worden aan cellen van het afweersysteem

T cellen

T cellen kunnen gepresenteerde peptiden op het celoppervlak van tumorcellen herkennen met behulp van een bepaald eiwit, de T cel receptor (TCR). Elke T cel heeft één TCR waarmee het één soort tumorpeptide kan herkennen. Een verworven afweerreactie wordt in gang gezet op het moment dat een tumorcel, bijvoorbeeld door de opruimreactie van het aangeboren afweersysteem, afsterft en zijn tumorspecifieke eiwitten in de bloedbaan vrij komen. De dendritische cellen nemen de vrijgekomen tumorspecifieke eiwitstructuren op en presenteren deze in de vorm van tumorspecifieke peptiden via de HLA moleculen op hun celoppervlak aan de T cellen. Dit resulteert in activatie en vermenigvuldiging van de tumorspecifieke T cellen en uit één tumorspecifieke T cel ontstaan vervolgens meerdere tumorspecifieke T cellen. Deze tumorspecifieke T cellen verlaten vervolgens de lymfklier om op zoek te gaan naar tumorcellen met dezelfde tumorpeptiden op hun celoppervlak. Op het moment dat een tumorspecifieke T cel een via de HLA moleculen gepresenteerde tumorpeptide herkent, wordt de tumorspecifieke T cel opnieuw geactiveerd. In plaats van zich te delen, scheidt de tumorspecifieke T cel cytotoxische granula uit, welke de tumorcel vernietigen. T cellen worden onderverdeeld in cytotoxische T cellen en helper T cellen. Cytotoxische T cellen herkennen peptiden die via HLA klasse I moleculen worden gepresenteerd, zijn cytotoxisch en kunnen met hun toxische granula tumorcellen vernietigen. Helper T cellen herkennen peptiden die via de HLA klasse II moleculen worden gepresenteerd en ondersteunen de cytotoxische T cellen, dendritische cellen en B

cellen bij het uitvoeren van hun functie. Daarnaast spelen helper T cellen een rol bij de "geheugenvorming" (memory) van het verworven afweersysteem. Dit is belangrijk, aangezien bij een eerste contact met een ziekteverwekker, het afweersysteem pas na enkele dagen op gang komt. Bij een tweede contact wordt het "geheugen" geactiveerd en verloopt de afweerreactie sneller. De memory van het afweersysteem is een belangrijke voorwaarde voor het langdurig "aanslaan" van tumorspecifieke immuuntherapie bij patiënten met kanker.

B cellen

Via de helper T cellen worden de B cellen aangezet tot de productie van antilichamen. Antilichamen zijn eiwitstructuren die onder andere specifieke eiwitten herkennen op het celoppervlak van ziekteverwekkers, geïnfecteerde cellen en tumorcellen. Op het moment dat antilichamen aan kankereiwitten binden, worden de kankercellen voor het afweersysteem zichtbaar en worden deze opgeruimd. Daarnaast stimuleren B cellen op hun beurt de T cellen doordat ze, net zoals dendritische cellen, antigenen opnemen en als peptiden via de op hun celoppervlak bevindende HLA klasse II moleculen aan de T cellen aanbieden.

Co-stimulatie

Een optimale T cel activatie ligt aan de basis van een optimale afweerreactie. T cel activatie is in de eerste plaats afhankelijk van de via de HLA moleculen gepresenteerde antigenen en in de tweede plaats afhankelijk van co-stimulatie. Co-stimulatie vindt plaats via unieke receptor-ligand combinaties en draagt bij aan het activeren en vermenigvuldigen van afweercellen of juist aan het afremmen van afweercellen, bijvoorbeeld om te voorkomen dat lichaamseigen cellen worden gedood (auto-immuunziekten). Zonder co-stimulatie kan het afweersysteem niet functioneren. Een belangrijke rol bij het herkennen en bestrijden van tumorcellen wordt vervuld door co-stimulatie-specifieke receptor-ligand interacties. Daarom staan co-stimulatie moleculen momenteel in de belangstelling van artsen en wetenschappers.

In dit proefschrift wordt veel aandacht besteed aan het co-stimulatie molecuul 4-1BB (CD137) omdat is aangetoond dat dit molecuul zowel een rol speelt bij tumor-specifieke afweerreacties als ook bij het ontstaan van verschillende auto-immuunziekten. De CD137-specifieke co-stimulatie receptor bevindt zich bij mensen op het celoppervlak van geactiveerde T cellen, NK cellen (dit proefschrift) en B cellen (dit proefschrift), terwijl de ligand (CD137L) zich op het celoppervlak van monocyten, dendritische cellen, B cellen en natural killer-like T (NKT) cellen

bevindt. CD137-CD137L interacties stimuleren de vermenigvuldiging en verlengen de levensduur van T cellen. Daarnaast spelen CD137-CD137L interacties een rol in de NK celspecifieke afweer tegen tumorcellen.

Actuele mogelijkheden van tumorspecifieke immuuntherapie

Antilichamen

Antilichamen zijn opgebouwd uit twee delen, een Fab-gedeelte en een Fc-gedeelte. Het Fab-gedeelte is antigeenspecifiek en kan één tumorspecifieke structuur op het celoppervlak van de tumorcel herkennen. Het Fc-gedeelte van een antilichaam kan door NK cellen via de Fc-receptor gebonden worden waardoor vervolgens antilichaamafhankelijke cel cytotoxiciteit (ADCC) gestimuleerd wordt. Antilichamen hebben dus grofweg drie functies: (i) door het binden aan tumorspecifieke structuren op het celoppervlak van de tumorcel wordt de tumorcel zichtbaar voor het afweersysteem en kan vervolgens vernietigd worden door NK cel-gemedieerde ADCC, (ii) door het binden aan tumorspecifieke structuren op het celoppervlak wordt de groei-impuls van de tumorcel geremd en (iii) door het binden aan specifieke structuren op het celoppervlak van afweercellen worden deze geactiveerd (bijvoorbeeld bij CD137-specifieke co-stimulatie) of juist geremd (bij het voorkomen van auto-immuunziekten).

Cytokinen

Cytokinen zijn eiwitten die het afweersysteem en mogelijk ook de al bestaande afweerreactie tegen tumorcellen kunnen activeren.

Vaccinatie

Tumor-specifieke vaccinatie was in eerste instantie gericht op de presentatie van tumorspecifieke eiwitstructuren aan T cellen door (i) tumorspecifieke peptiden, (ii) tumorspecifieke eiwitten, (iii) bestraalde tumor cellen of (iv) tumor cel lysaten te injecteren. De gedachte was dat op deze manier de dendritische cellen de geïnjecteerde eiwitstructuren zouden opnemen en presenteren aan de T cellen. Later werden behandelingsstrategieën ontwikkeld waarbij dendritische cellen beladen met tumorspecifieke eiwitstructuren of getransfecteerd met tumorspecifiek materiaal werden geïnjecteerd. In dit geval konden de opgenomen tumorspecifieke eiwitstructuren via de op de dendritische cel aanwezige HLA moleculen aan het afweersysteem gepresenteerd worden. Deze strategie vormde in 2010 de basis voor de eerste op afweercellen gebaseerde behandeling van patiënten met prostaatkanker (Sipuleucel-T; Provenge®).

Adoptieve cel transfer

Bij adoptieve cel therapie (ACT) worden afweercellen uit de bloedbaan geïsoleerd en buiten het lichaam achtereenvolgens geselecteerd, geactiveerd en vermenigvuldigd. Nadat de betreffende afweercellen voldoende geactiveerd en vermenigvuldigd zijn, worden ze via een infuus direct in de bloedbaan van de patiënt terug gegeven. Op dit moment ligt het zwaartepunt bij de vermenigvuldiging en infusie van T cellen (al dan niet genetisch gemodificeerd). De interesse in selectie, activatie en vermenigvuldiging van NK cellen voor adoptieve NK cel therapie staat echter ook in de belangstelling (dit proefschrift).

Hindernissen bij de toepassing van tumorspecifieke immuuntherapie

Voor een succesvolle vernietiging van tumorcellen is het noodzakelijk dat het afweersysteem voldoende geactiveerd wordt én dat de geactiveerde afweercellen de tumorcellen herkennen. Helaas is aangetoond dat de afweercellen van mensen met kanker afwijken van de afweercellen van mensen zonder kanker. Kankerpatiënten hebben bijvoorbeeld minder co-stimulatie en HLA klasse II moleculen op het celoppervlak van hun dendritische cellen. Ook is aangetoond dat de TCR bij kankerpatiënten minder goed functioneert en dat kankerpatiënten verhoogde aantallen van afweerremmende T cellen bezitten (regulatoire T cellen). Daarnaast bezitten tumorcellen de mogelijkheid om zich tegen het afweersysteem te beschermen door zelf afweerreacties af te remmen of door zich onzichtbaar te maken. Dit gebeurt bijvoorbeeld door het uitscheiden van afweerremmende stoffen door de tumorcel, door het tot expressie brengen (up-regulatie) van afweerremmende ligands op het celoppervlak van de tumorcel en door een verminderde expressie van HLA moleculen (down-regulatie) op het celoppervlak van de tumorcel.

Het is dus duidelijk dat tumorspecifieke immuuntherapie verschillende afwijkingen in het afweersysteem moet omzeilen, alvorens het afweersysteem te kunnen activeren. Daarnaast is het aannemelijk dat het na elkaar of gelijktijdig toepassen van verschillende vormen van immuuntherapie een grotere kans op genezing biedt.

Dit proefschrift

Op dit moment is tumorspecifieke immuuntherapie vooral gericht op de vernietiging van tumorcellen door antilichamen en op de activatie van cytotoxische T cellen binnen de verworven afweer. Relatief weinig therapieën richten zich op de

optimalisatie van co-stimulatie, de aangeboren afweer en helper T cellen. Om de invalshoeken van tumorspecifieke immuuntherapie verder uit te breiden, mede met het doel om bestaande immuuntherapeutische mogelijkheden verder te optimaliseren, wordt in dit proefschrift de therapeutische potentie van CD137-specifieke co-stimulatie (deel A) en NK cel therapie (deel B) voor de behandeling van patiënten met solide tumoren beschreven. Daarnaast worden de eerste klinische resultaten van vaccinatie met HLA klasse I en II specifieke MAGE- en HPV-peptiden bij patiënten met hoofd-hals tumoren besproken.

Deel A: CD137-specifieke co-stimulate

Het eerste deel van dit proefschrift richt zich op de rol van CD137 op menselijke NK cellen, B cellen en de toepassing van diermodellen op de translatie van CD137-specifieke antilichamen naar bruikbare medicijnen in klinische studies voor patiënten met solide tumoren.

Hoofdstuk 2

Gebaseerd op (i) de in muismodellen aangetoonde rol van CD137-specifieke antilichamen in de afweerreactie tegen tumorcellen, (ii) de cruciale rol van muizen NK cellen in CD137-gemediieerde afweerreacties tegen tumorcellen en (iii) de belangrijke rol van Fc-receptoren in het functioneren van antilichamen in het algemeen, hebben we in **hoofdstuk 2** van dit proefschrift de uitwerking van CD137- specifieke antilichamen op menselijke NK cellen bestudeerd. Vanwege de aangetoonde rol van NK cellen in de door CD137-specifieke antilichamen gestimuleerde tumorspecifieke afweerreactie in muizen, gingen we ervan uit dat Interleukine-(IL)2 geactiveerde menselijke NK cellen het molecuul CD137 tot expressie zouden brengen. Ook gingen we ervan uit dat binding van het CD137-specifieke antilichaam aan het molecuul CD137 zou leiden tot NK cel gemedieerde cytotoxiciteit. De resultaten lieten echter zien dat menselijke, IL-2 geactiveerde NK cellen geen tot zeer weinig CD137 tot expressie brengen. In tegendeel, voor het tot expressie brengen van CD137 op menselijke NK cellen, diende de Fc-receptor van de NK cel het Fc-gedeelte van het antilichaam te binden. Daarnaast toonden we aan dat het tot expressie brengen van CD137 niet afhankelijk is van het Fab-gedeelte van het antilichaam en dat de hoeveelheid CD137 expressie geassocieerd is met het aantal aanwezige glucosemoleculen (suikers) in het Fc-gedeelte van een antilichaam. Deze bevindingen zijn belangrijk voor de juiste interpretatie van gepubliceerde experimenten met CD137-specifieke antilichamen in muizen. CD137-specifieke afweerreacties, welke geïnterpreteerd werden als Fab-gemedieerd kunnen door onze resultaten evenwel Fc-gemedieerd zijn.

Hoofdstuk 3

Op basis van de bevindingen in hoofdstuk 2 ontstond er twijfel over de relevantie van muismodellen om menselijke afweerreacties op CD137-specifieke antilichamen te voorspellen. B cellen spelen een duidelijke rol in de afweer tegen tumorcellen en aangenomen wordt dat de stimulatie van CD137 op T cellen verantwoordelijk is voor de veranderingen in B cel-gemedieerde afweer en de overleving van B cellen. Er is echter weinig bekend over de directe rol van CD137-gemedieerde co-stimulatie op menselijke B cellen. Hier is ook van belang dat muizen B cellen geen CD137 tot expressie brengen en dat menselijke B cellen CD137 tot expressie brengen na stimulatie met antilichamen. In **hoofdstuk 3** hebben we eerst geëvalueerd welke signalen verantwoordelijk zijn voor de expressie van CD137 op menselijke B cellen. Daarna hebben we bekeken welke invloed CD137 heeft op de functie van menselijke B cellen. Onze experimenten toonden aan dat antilichaamgeactiveerde menselijke B cellen, CD137 tot expressie brengen op het moment dat de B cel receptor (BCR) gestimuleerd wordt. Stimulatie van het CD137 molecuul op menselijke B cellen leidde tot B cel deling en een betere B cel overleving. Uit deze experimenten konden we concluderen dat de expressie van CD137 op menselijke B cellen zeer nauwkeurig geregeld wordt, afhankelijk is van stimulatie met antilichamen en dat de BCR de uiteindelijke expressie van CD137 op menselijke B cellen bepaald.

Hoofdstuk 4

Nieuwe behandelingsmogelijkheden mogen alleen aan mensen met kanker worden aangeboden als de uitwerking van het nieuwe medicijn op proefdieren als "veilig" kan worden aangenomen. Normaal gesproken moeten nieuwe medicijnen zowel op muizen als apen op veiligheid worden getest. Om in proefdieren te kunnen vaststellen of een medicijn als "veilig" kan worden beschouwd, is het noodzakelijk dat de afweercellen van het proefdier of de tumor van het proefdier het te testen medicament herkent en kan binden. De in hoofdstuk 2 en 3 aangetoonde verschillen in CD137 expressie tussen muizen en menselijke NK cellen en B cellen brengen duidelijk de grenzen van het voorspellend vermogen van muismodellen op de invloed van CD137-specifieke co-stimulatie op het menselijke afweersysteem naar voren. Om vast te kunnen stellen welk proefdier voor het testen van de CD137-specifieke antilichamen geschikt is, hebben we in **hoofdstuk 4** gekeken of de CD137 moleculen op het celoppervlak van witte bloedcellen van mensen, resusapen en bavianen het CD137-specifieke antilichaam herkenden en konden binden. Dit bleek een waardevolle test, aangezien het CD137-specifieke antilichaam het CD137 molecuul op de witte bloedcellen van apen niet bond. Dit

was uiteindelijk terug te voeren op een klein, maar essentieel, genetisch verschil tussen witte bloedcellen van mensen en apen. Alleen chimpansees hebben de gelijke genetische CD137 codering en onderzoek met deze apen is streng gelimiteerd.

Deel B: NK cel therapie

Hoofdstuk 5

Voor celtherapie met NK cellen is het essentieel om deze cellen buiten het lichaam te kunnen vermenigvuldigen. Eerder onderzoek heeft aangetoond dat CD137-CD137L interacties het vermenigvuldigen van NK cellen buiten het menselijk lichaam stimuleert. In **hoofdstuk 5** beschrijven we de succesvolle vermenigvuldiging (expansion) van NK cellen uit het bloed van patienten met maagkanker, longkanker, darmkanker en leverkanker. Ook toonden we aan dat ze NK celspecifieke cytotoxische moleculen op hun celoppervlak tot expressie brengen, waarmee ze in staat zijn om lichaamseigen tumorcellen te herkennen en op te ruimen. Ook lieten de experimenten in dit hoofdstuk zien dat de vermenigvuldigde NK cellen in staat zijn tot ADCC, aangezien hun cytotoxisch vermogen duidelijk toenam in de aanwezigheid van een antilichaam welke het molecuul EGFR op tumorcellen herkent en bindt. De op het celoppervlak van de NK cel tot expressie gebrachte cytotoxische moleculen speelden hierbij een cruciale rol. Daarnaast werd een eerste stap naar de klinische toepassing van deze cellen in NK celtherapie gezet door aan te tonen dat een reeds bestaande techniek voor de isolatie van monocyten ter voorbereiding van celtherapie met dendritische cellen ook toegepast kan worden voor het op grote schaal produceren van cytotoxische NK cellen voor NK celtherapie. Tevens kan de gecombineerde toepassing van de NK cellen, beschreven in dit hoofdstuk, met al bestaande tumorspecifieke antilichamen het therapie-effect van deze antilichamen versterken.

Hoofdstuk 6

Voor NK cel gemedieerde ADCC is de interactie tussen het Fc-gedeelte van een antilichaam en de Fc-receptor op de NK cel essentieel. Eerder onderzoek heeft aangetoond dat specifieke genetische veranderingen (polymorphismen) in de Fc-receptor geassocieerd zijn met een verhoogde kans op slagen van een behandeling met antilichamen bij patiënten met een lymfoom. Omdat in hoofdstuk 5 kon worden aangetoond dat door ADCC de cytotoxiciteit van de vermenigvuldigde NK cellen duidelijk toenam, hebben we in **hoofdstuk 6** bepaald of de eerder aangetoonde Fc-polymorphismen bij patiënten met bloedkanker ook een rol spelen bij patiënten met solide tumoren. Om dit te onderzoeken hebben we in hoofdstuk 6

het effect van verschillende Fc-polymorphismen op EGFR-gemedieerde ADCC in hoofd-hals tumoren met elkaar vergeleken. Uit de experimenten bleek dat bij patiënten met hoofd-hals tumoren Fc-polymorphismen niet alleen een rol spelen bij ADCC maar ook bij het antigeen-onfhankelijke vermogen van NK cellen om tumorcellen te herkennen en te vernietigen. Dit is een belangrijke bevinding, aangezien op deze manier vooraf bepaald kan worden bij welke patiënten een behandeling met tumorspecifieke antilichamen of NK cellen een hoge kans van slagen heeft.

Deel C: Van de theorie naar de praktijk

In eerste instantie werd aangenomen dat HLA klasse I moleculen en cytotoxische T cellen onmisbaar zijn in de verworven afweer tegen tumorcellen. Door het op grote schaal "falen" van vaccinatiestudies, waarbij alleen de cytotoxische T cellen geactiveerd werden, werd de noodzaak tot het activeren van zowel cytotoxische T cellen als ook helper T cellen aannemelijk. **Hoofdstuk 7** bevat de eerste klinische resultaten van de behandeling van patiënten met hoofd-hals tumoren met Trojan-peptiden. Trojan-peptiden zijn opgebouwd uit verschillende HLA klasse I en II peptiden met het doel cytotoxische T cellen en helper T cellen te activeren. Bij vier van de vijf behandelde patiënten konden we aantonen dat de helper T cellen door vaccinatie met de Trojan-peptiden geactiveerd werden. Daarnaast werden bij één patiënt ook de cytotoxische T cellen geactiveerd. De vaccinaties werden door de patiënten goed verdragen en drie van de vijf behandelde patiënten leefde langer dan 24 maanden in een situatie waar normaal gesproken de levensverwachting onder de zes maanden ligt.

Verschillende aspecten van deze klinische studie zijn van belang voor de toekomst van tumorspecifieke immuuntherapie. Zoals eerder beschreven moeten verschillende afwijkingen in het afweersysteem omzeilt worden, alvorens het afweersysteem geactiveerd kan worden. De Trojan-peptiden zijn in eerste instantie gericht op de activatie van het afweersysteem en omzeilen niet de eventueel bestaande afwijkingen in de afweercellen van een patiënt. Aangezien het aannemelijk is dat alleen het, na elkaar of gelijktijdig, toepassen van verschillende vormen van immuuntherapie een kans op genezing biedt, kan de werking van de Trojan-peptiden geoptimaliseerd worden door bijvoorbeeld gelijktijdig met CD137-specifieke antilichamen te behandelen. Ook het aantal aanwezige tumorcellen bepaald sterk de kans van slagen van tumorspecifieke immuuntherapie aangezien een patient met een groot aantal tumorcellen (uitzaaiingen) zeer waarschijnlijk ook meer tumorspecifieke T cellen nodig heeft om alle tumorcellen

te kunnen vernietigen. Vaccineren in een vroeg, nog beter "tumor vrij", stadium is dus noodzakelijk.

Conclusies

In de afgelopen 20 jaar heeft tumorspecifieke immuuntherapie een plaats ingenomen in de behandeling van patiënten met kanker. De toegenomen duidelijkheid over de interactie tussen het afweersysteem en tumorcellen heeft ertoe bijgedragen dat op dit moment verschillende potentiële behandelingsmogelijkheden in klinische studies aan patiënten met kanker worden aangeboden (www.clinicaltrials.gov). Samengevat bouwt dit proefschrift de bestaande invalshoeken van tumorspecifieke immuuntherapie verder uit. Het beschrijft de potentie, uitdagingen in klinische translatie en mogelijkheden tot het gecombineerd inzetten van CD137-specifieke co-stimulatie en op NK cellen gebaseerde celtherapie met het doel bestaande mogelijkheden verder te optimaliseren. Met het gegeven dat iedere patiënt met kanker unieke afwijkingen in het afweersysteem heeft, waardoor in de eerste plaats de ziekte kanker heeft kunnen ontstaan, is het aannemelijk dat alleen het gecombineerd inzetten van vaccinatie, adoptieve cel therapie en manipulatie van het afweersysteem door (co-stimulatie-specifieke) cytokinen en antilichamen, kan leiden tot een succesvolle behandeling bij een groot deel van alle kankerpatiënten.

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Acknowledgements

This incredible journey started in May 2004 on a hot Maltese beach with Co explaining his scientific work, accomplished in Boston, by showing me some power point slides. At that time, I was clueless about my own goals in medicine and the American adventure Co was proposing in that sunny atmosphere, seemed a nice escape from me having to decide what to do. The picture taken at that beach has been travelling with us ever since, and symbolizes the start of our ongoing journey together in life. With my PhD defense just a few weeks ahead, the time has come to write the final words of this thesis. Being a physician interested in research is almost "a way of life" in which work and private matters sometimes are difficult to separate. In that regard, many people contributed to this thesis.

Prof. Nijman, dear Hans, we first met at the AACR tumor immunology meeting in Miami, Florida, in December 2006. The research just started to come along and you suggested considering a PhD under the premise of one published paper every year during my research fellowship. I had none at that time…We met again during the AACR tumor immunology meeting in December 2008 and, to my own surprise, a PhD did not seem like a joke anymore. I would like to thank you for your enthusiasm and confidence at an early stage in my research career. You gave me a goal to work for and this securely linked a "no" to the "shall-I-throw-the-towel" question. I can't believe I made it this far!

Prof. Mann, dear Dean, you offered a position in your lab at a time I could barely pronounce the word "immunology". I would like to thank you for your trust, faith and the unlimited opportunity to explore and learn. In addition, I would like to thank you, Petra, Emma and Alexandra for all the family-moments we have shared.

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Time is scarce at the highest level of academia. Therefore, I would like to thank Prof. van der Burg, Prof. Roodenburg and Prof. Schuler for their careful review of this thesis.

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Starting my research fellowship would not have been possible without the help of Prof. Suzanne Ostrand-Rosenberg and Prof. Robert Rosenberg. Dear Sue and Bob, thank you for your great hospitality during all those years. Sue, you played an important role in finding job opportunities at the University of Maryland and after moving, you both helped us getting settled by offering a place to stay during our house- and car hunt. In addition, you always offered a listening ear during difficult moments. These conversations have definitely improved my personal strength.

The laboratories, in which the presented work has been carried out, have been the backbone of my American life and gave me the opportunity to grow as a scientist and person.
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Leaving the US, I had to say goodbye to our very close friends Lin Wei and Zhaorong, Isa Diaz-Mendez, Joaquin Muriel-Gonzalez, Greg Harris and Kristina Harris.
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Dear Isa and Joaquin, you are a huge example to me. I have deep respect for the way you are living your life and can only wish to become a fraction of the parents you are. I would like to thank you, David and Laura, for all the warm moments we shared. They will never be forgotten.
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"Time flies when you are having fun" and it has been over seven years that I left the Netherlands. I would like to thank my dear Dutch friends Machteld Jansen Schoonhoven, Attie Kuiken en Sanne de Vogel for their endless support and understanding. Dear Attie, Machteld and Sanne, I think we have really proven that

long-distance friendships do last! The same holds true for the friendship with Luciana Conde, Paula DeFrancesco and Guillermina Fernandez in Adolfo Gonzales Chaves, Argentina. Querido Lu, Pau y Guille, las quiero mucho.

Research fellow in the USA, resident in Germany, it sometimes felt more like an emotion-driven rollercoaster than a well-chosen way of life. In that regard, I would like to thank my paranymfen, whose contributions expand far beyond this day in September.
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"Happiness is having a large, loving, caring, close-knit family in another city."
George Burns (1896-1996)

Dear Bosch family, dear pa en ma, Mariëtte, Roeland, Luuk, Elizabeth and Sophie, thank you for welcoming me in your family and providing a second home. I hope we will share many more warm moments sitting around a fire place!

Dear Marije and Driek, I am proud to be your sister! Marije, your sense for unconditional care is something to be jealous of. Driek, you introduced me to Siddhartha, and showed me the way towards intellectual stability. Thank you both for reaching out when things get really tough.

Dear mama and papa, I have deep respect for the way you live your own lifes and how perfectly they seem to fit within each other. You never question or judge but instead, wait and see while offering a warm base to return to if things get out of control. Since I met Co, the "crazy" factor quadrupled, yet you accepted him as one of your own. Thank you for letting us live our own life!

Finally, while thinking of the final words of this thesis, my eyes slowly start to get wet. Dear Co, I dedicate this thesis to you.

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List of publications and curriculum vitae

List of publications

Voskens CJ, Cavallaro A, Erdmann M, Dippel O, Kaempgen E, Schuler-Thurner B, Schuler G, Heinzerling L. Anti-CTLA-4 induced regression of spinal cord metastases in association with renal failure, atypical pneumonia, vision loss and hearing loss. *Journal of Clinical Oncology* accepted

Voskens CJ, Sewell D, Hertzano R, DeSanto J, Rollins S, Lee M, Taylor R, Wolf J, Suntharalingam M, Gastman B, Papadimitriou JC, Lu C, Tan M, Morales R, Cullen K, Celis E, Mann D, Strome SE. Trojan vaccines induce MAGE-A3 and HPV-16 immunity in patients with head and neck cancer. *In Press*

Voskens CJ, Watanabe R, Rollins S, Campana D, Hasumi K, Mann DL. Ex-vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity. *J Exp Clin Cancer Res.* 2010 Oct 11;29:134

Zhang X, **Voskens CJ**, Sallin M, Maniar A, Montes CL, Zhang Y, Lin W, Li G, Burch E, Tan M, Hertzano R, Chapoval AI, Tamada K, Gastman BR, Schulze DH, Strome SE. CD137 promotes proliferation and survival of human B cells. *J Immunol.* 2010 Jan 15;184(2):787-95

Voskens CJ, Strome SE, Sewell DA. Synthetic peptide-based cancer vaccines: lessons learned and hurdles to overcome. *Curr Mol Med.* 2009 Aug;9(6):683-93. Review.

Chan SL, **Voskens CJ**, Lin W, Schindler DG, Azimzadeh A, Wang LX, Taylor RJ, Strome SE, Schulze DH. Epitope mapping of a chimeric CD137 mAb: a necessary step for assessing the biologic relevance of non-human primate models. *J Mol Recognit.* 2009 May-Jun;22(3):242-9

Taylor RJ, Chan SL, Wood A, **Voskens CJ**, Wolf JS, Lin W, Chapoval A, Schulze DH, Tian G, Strome SE. FcgammaRIIIa polymorphisms and cetuximab induced cytotoxicity in squamous cell carcinoma of the head and neck. *Cancer Immunol Immunother.* 2009 Jul;58(7):997-1006

Lin W*, **Voskens CJ***, Zhang X, Schindler DG, Wood A, Burch E, Wei Y, Chen L, Tian G, Tamada K, Wang LX, Schulze DH, Mann D, Strome SE. Fc-dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies. *Blood* 2008 Aug 1;112(3):699-707
*Authors contributed equally to this study

Curriculum vitae

The author of this thesis was born on October 6th, 1977 in Zeist, the Netherlands. She graduated from high school (Christelijk College Zeist) in 1996 and in the same year moved to the rural town of Adolfo Gonzales Chaves, Argentina, as a participant in Rotary's International Youth Exchange Program 1996/1997.

In 1997, she started to study Biological and Medical Sciences at Leiden University and in 1998 she was able to join medical school at the same university. During medical school she worked as a transplantation officer and office administrator at the BIS Foundation, a non-profit organization that operates as an intermediary in the donation and allocation of corneas, skin, heart valves and bone tissue for transplantation purposes.

In 2002, the author conducted her scientific graduation project at the Wellcome Trust Research Laboratories, Blantyre, Malawi under supervision of Dr. L. van Lieshout and Dr. M. Boele van Hensbroek. This project evaluated different techniques to diagnose hookworm in stool, as part of a large research project on etiology, pathogenesis and long-term outcome on severe anemia in Malawian children.

Returning to the Netherlands, she finished her clinical rotations (2003/2004) at the Leiden University Medical Center and affiliated hospitals resulting in a MD degree at the end of 2004.

In 2005 she started to work as a research fellow at the University of Maryland, Baltimore, USA, were immuno-monitoring studies of a phase I clinical trial on two peptide-based cancer vaccines were combined with preclinical studies on humanized antibodies and natural killer cells.

In 2009 the author returned to Europe and was awarded a training position in the department of Dermatology, University of Erlangen, Germany under supervision of Prof. G. Schuler. Here she currently works as a resident and, in addition, is involved in clinical studies on RNA transfected dendritic cells in melanoma patients as well as preclinical studies on the expansion of regulatory T cells for the treatment of inflammatory bowel disease. In April 2011 she received an award for best oral speaker during the 46th conference of the German Society of Dermatology. She is married to Co Bosch.

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Color figures, Supplemental figures and Supplemental tables

Chapter 2

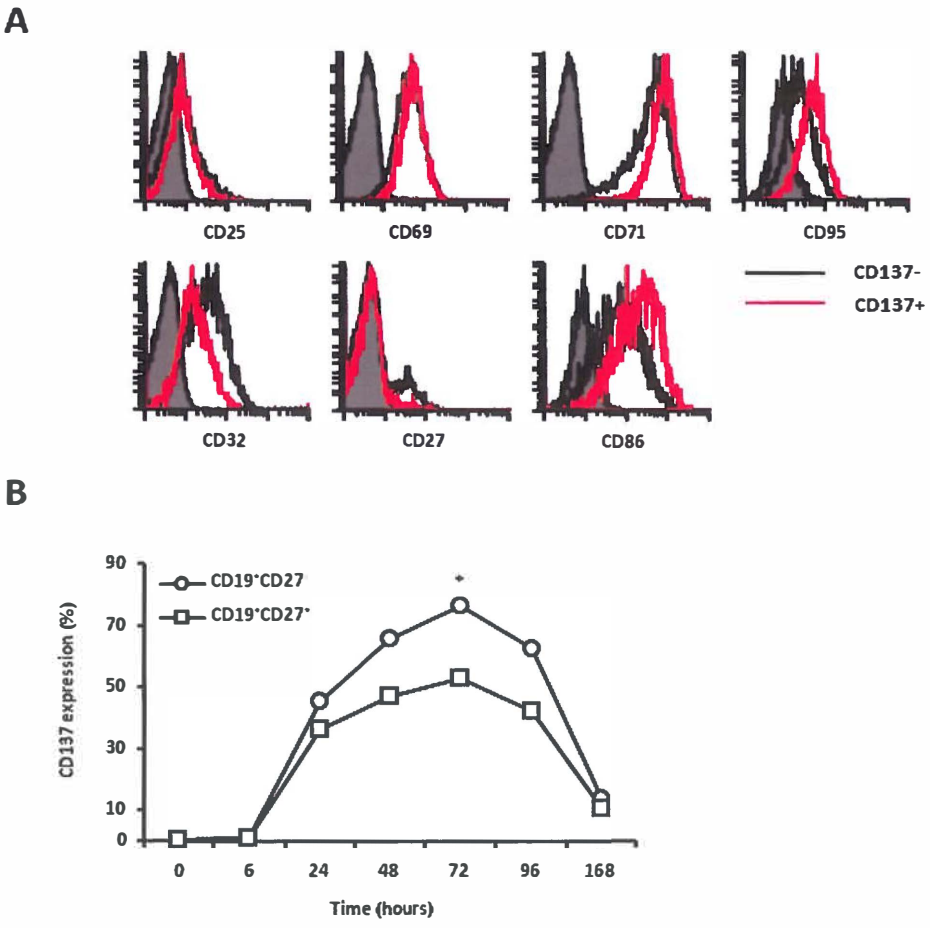


Figure 3. CD137 is preferentially expressed on activated B cells of naïve origin
Purified human B cells were activated with anti-Ig/anti-CD40. After 3 days, B cells were harvested and CD137 expressing B cells and non-CD137 expressing B cells were assessed for cell surface phenotype by flow cytometry **(A)**. Histograms show surface expression of indicated markers on $CD137^+$ B cells and $CD137^-$ B cells. Filled peaks represent isotype controls. Data are representative of 5 individual experiments. Purified human B cells were separated into naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells and subsequently stimulated with anti-Ig/anti-CD40. CD137 surface expression was determined at indicated time-points **(B)**. Data shown are representative of 5 individual experiments. *P<0.05.

Chapter 2

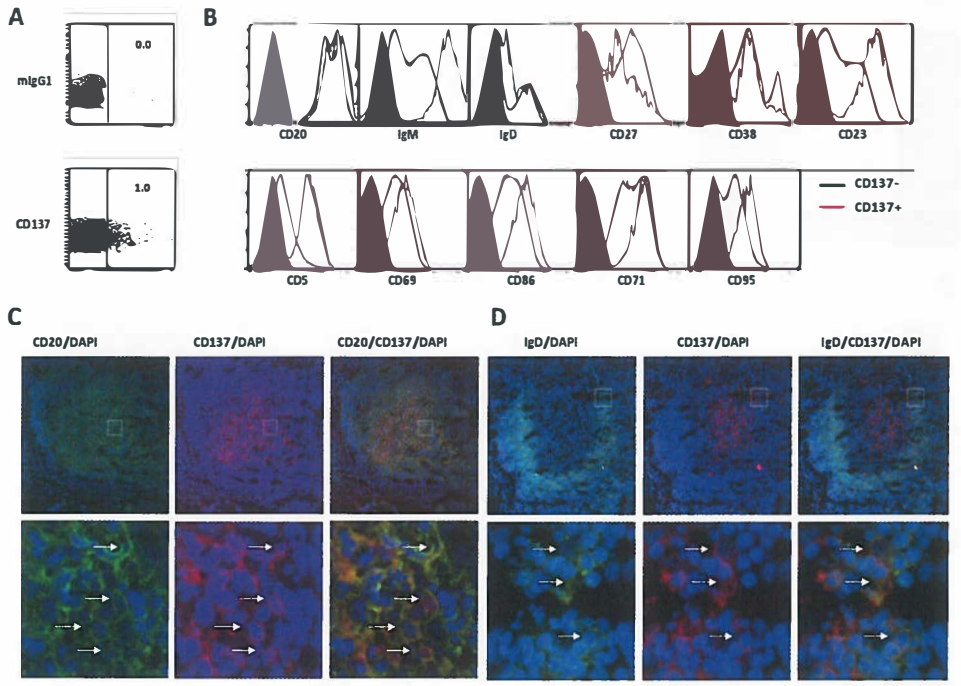


Figure 4. *Ex vivo* expression of CD137 in tonsillar B cells
Tonsillar mononuclear cells were analyzed for CD137 expression on B cells by FACS. B cells ($CD19^+CD3^-$) were gated and the percentage of CD137 expression on B cells is indicated in a dot plot **(A)**. Data are representative of 6 individual experiments from different donors. The phenotype of $CD137^+$ B cells was analyzed in comparison to $CD137^-$ B cells **(B)**. Filled peaks indicate isotype controls. Data are representative of 5 individual experiments. Three-color immunohistochemistry of human tonsil sections was used to identify the localization of CD137 expressing B cells. The upper panel shows a tonsillar germinal center (original magnification ×20; CD20, green; CD137, red; DAPI, blue) **(C)** and follicular mantle zone (IgD, green; CD137, red; DAPI, blue) B cells **(D)**. The indicated area from germinal centers and follicular mantle zones were enlarged to identify the $CD20^+/CD137^+$ and $IgD^+/CD137^+$ cells (lower panel).

Chapter 7

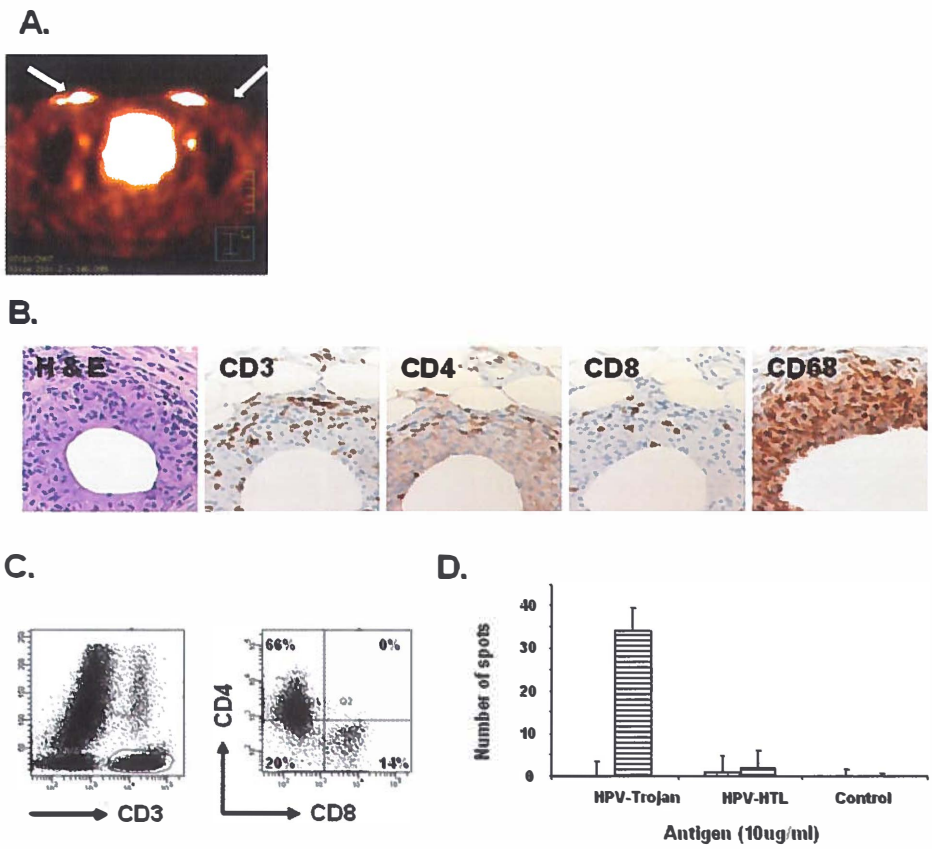


Figure 5. Trojan-specific precursor frequency at the injection site exceeds precursor frequency in the peripheral circulation. Frozen section slides were generated from a granuloma derived from the site of immunization and stained by immunohistochemistry with a variety of leukocyte-specific markers. The granuloma was biopsied 19 days after the second vaccination. PET scan revealing high levels of inflammation at the injection sites as indicated by arrows **(A)**; high numbers of antigen-presenting cells ($CD68^+$) and $CD3^+$ T cells infiltrated the site of immunization **(B)**; part of the granuloma was enzymatically digested into a single cell suspension and evaluated for Trojan-vaccine specificity **(C)**. As measured by flow cytometry, the majority of $CD3^+$ T cells in the granuloma were found to be $CD4^+$ cells. Next, cells were directly *ex vivo* evaluated for Trojan-specificity by IFNγ Elispot. High numbers of Trojan-specific cells were found at the site of injection **(D)**. In contrast, at the same time-interval, no Trojan-specific responses were detected in cells (PBMC) derived from the peripheral circulation. Number of spots per 50,000 cells (granuloma) and 100,000 cells (PBMC) is shown. Error bars represent SD from triplicate wells. * $P<0.05$

Chapter 7

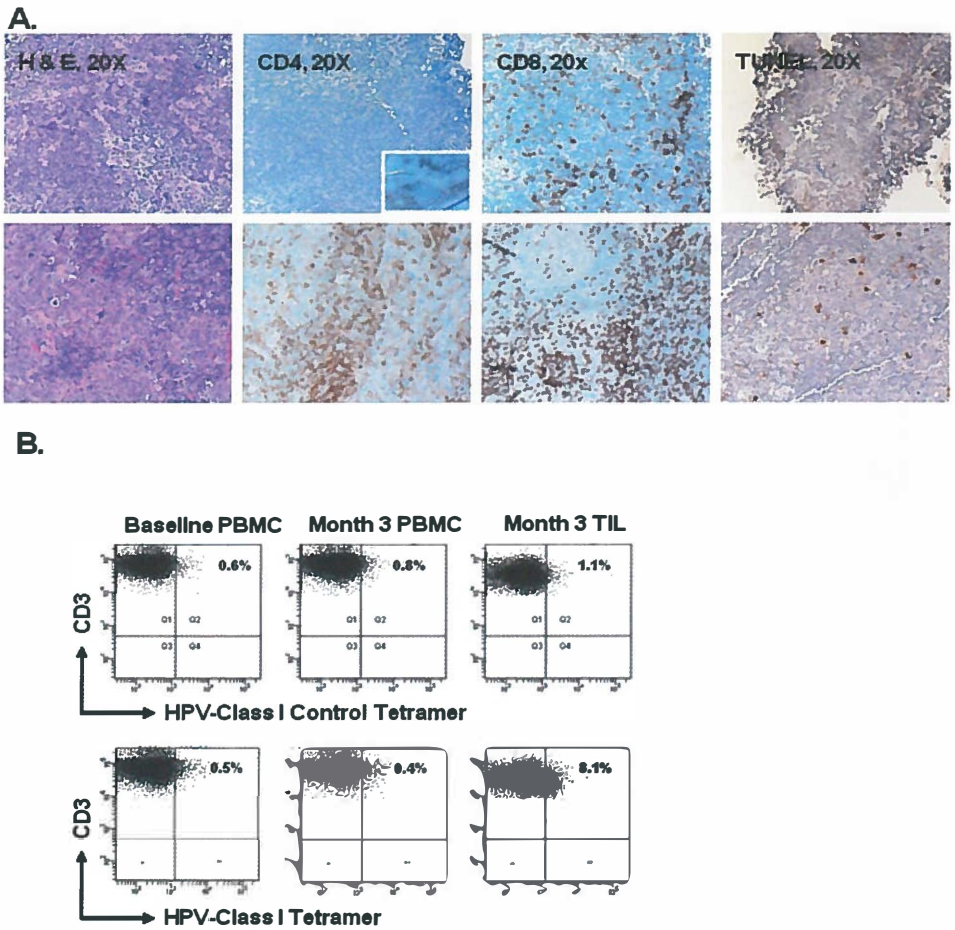
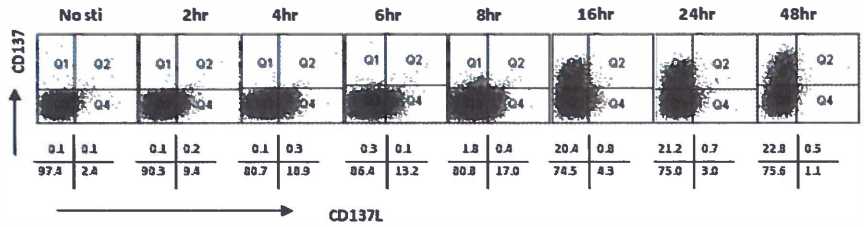
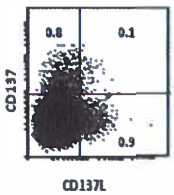


Figure 6. CD8$^+$ and CD4$^+$ T cells infiltrate the tumor microenvironment during immunization and a subset appears to be HPV-specific. Photomicrographs of immunohistochemical staining of tumor tissue harvested before and after immunization with HPV-Trojan vaccine. High numbers of CD8$^+$ and CD4$^+$ cells were found in the tumor micro-environment after immunization **(A)**. Representative sections from CD4 and CD8 staining of the neck metastasis slides before and three months after the last vaccination are shown. Positive controls were positive for all experiments (inset CD4). Part of the neck metastasis, collected at three months after immunization, was enzymatically digested into a single cell suspension (TIL). PBMC collected before and PBMC and TIL collected after three months of immunization were directly *ex vivo* stained with HPV-16 specific HLA-A*0201 tetramer and analyzed by flow cytometry for Trojan HLA-I specificity **(B)**. In comparison to PBMC collected at the same time-interval, TIL derived from the tumor micro-environment after 3 months of immunization were found to be HPV-16 specific, 0.4% and 8.1% respectively.

Chapter 2



Supplemental figure 1



Supplemental figure 2

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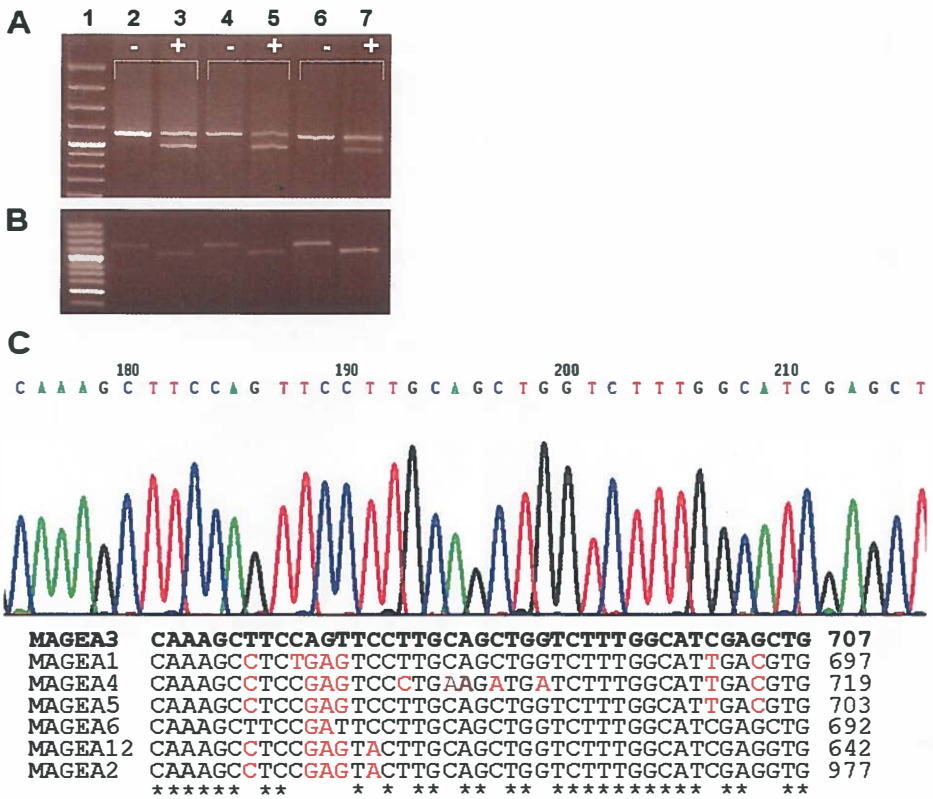

Supplemental figure 1. Specific Amplification of MAGE-A3. RNA was extracted from cell lines TU159 (lanes 2,3), 012SCC (lanes 4,5) and HW12 (lanes 6,7) which express multiple MAGE proteins. PCR was then performed with the previously published MAGE-A3 primers **(A)** or with primers MA3_F4 and MA3_R4 **(B)**. PCR products from both sets of primers were subjected to restriction digestion with *EcoR*I and then separated by agarose gel electrophoresis [(-) undigested, (+) digested]. PCR products that were amplified with MA3-F4 and MA3-R4 resulted in complete digestion of the product **(B)** while PCR products amplified by the previously published MAGE-A3 primers resulted in incomplete digestion, suggesting of non-specific amplification **(A)**. Direct sequencing of the MA3_F4 and MA3_R4 PCR products confirms the specificity of the amplified sequence as demonstrated by the chromatogram and multiple sequence analysis of the homologous MAGE genes are the region of the Trojan peptide **(C)**.

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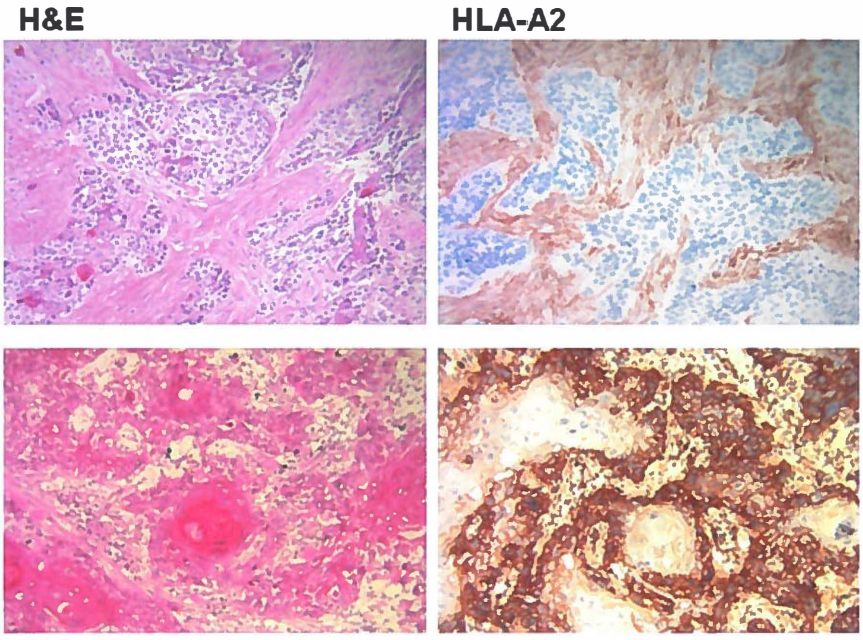

Supplemental figure 2. Loss of HLA-A*02 expression in SCCHN. (Top) H&E stain of the tumor shows sparse keratinization (left) and complete loss of HLA-A*02 staining, whereas the interstitial fibrous tissue shows intense staining (right). (Bottom) H&E stain of the tumor shows extensive keratinization (left) and intense tumor cell staining for HLA-A*02 (right).

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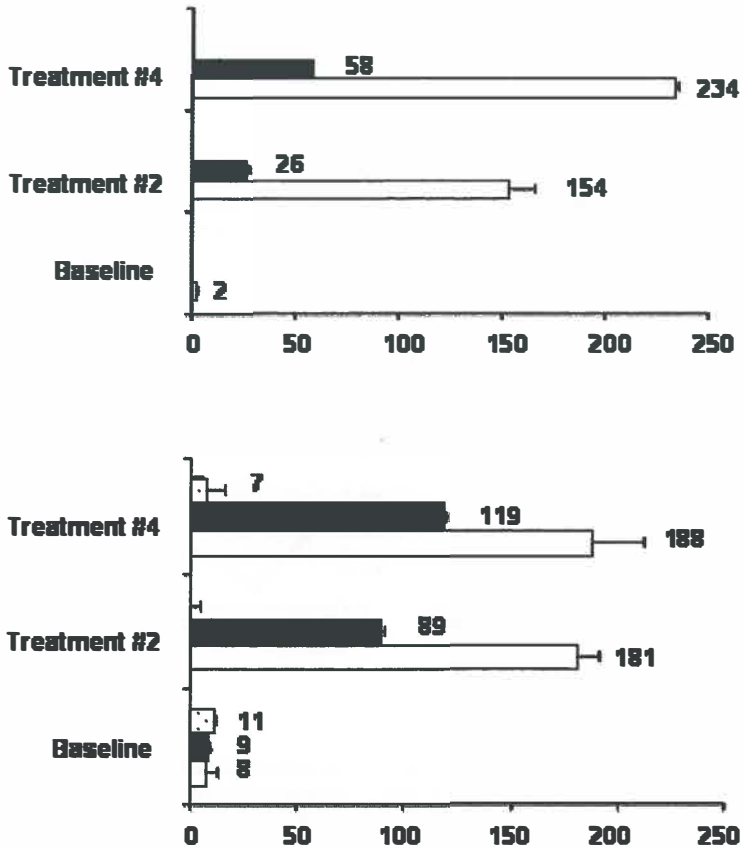

Supplemental figure 3. Quantitative immune responses. Quantitative immune responses as measured by re-stimulation Elispot of both patients who completed the full vaccination schedule are presented for MAGE-A3/patient 3 **(A)** and HPV-16/patient 5 **(B)**. White bars indicate responses against the Trojan vaccine; black bars indicate responses against the HTL-peptide; dotted bars indicate responses against the CTL peptides. Error bars represent SD from triplicate wells

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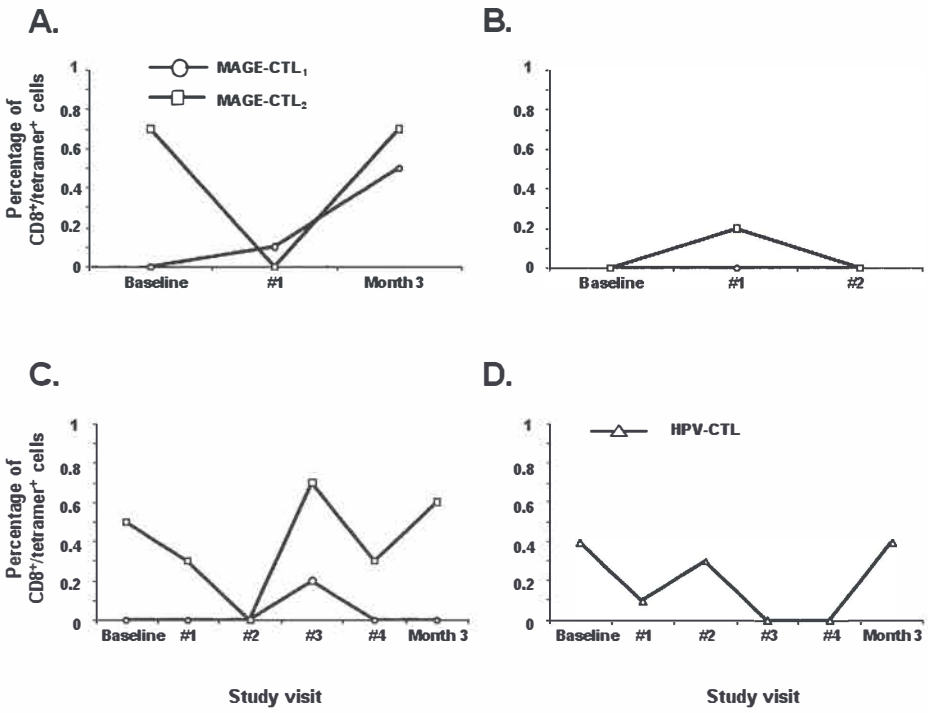

Supplemental figure 4. Trojan peptide-based vaccines fail to induce robust immunity to the HLA-I restricted epitope. Percentage of circulating MAGE-A3 and HPV-16 specific $CD8^+$ T cells were determined by staining of peripheral blood mononuclear cells obtained before and after immunization with control CTL (YIGEVLVSV), MAGE-CTL_1 (KVAELVHFL) and MAGE-CTL_2 (FLWGPRALV) or HPV-CTL (TLGIVZPI) specific tetramer. Percentage of vaccine-specific $CD8^+$ T cells was calculated by subtracting the number of cells which stained positive with control tetramer. Percentage of $CD8^+Tetramer^+$ T cells is shown at indicated time-points for patient 1 **(A)**, patient 2 **(B)**, patient 3 **(C)** and patient 5 **(D)**.

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Supplemental table 1 $CD3^+$, $CD8^+$ and total white blood cell counts before treatment

Value[a]	Patient				
	1	2	3	4[b]	5
WBC (K/mcL)	4.8	9.4	7.2	0.8	5.2
$CD3^+$ (%)	44.1	5.1	29.2	n/a	44.0
$CD3^+CD8^+$ (%)	42.	25.2	31.5	n/a	27.0

[a]WBC; white blood cell count (normal range 4.0-10.0 K/mcL)
[b]single use exemption, due to a secondary diagnosis of myelodysplastic syndrome
n/a, not applicable